Strategies to identify candidates for D variant genotyping

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Background. RhD variants have altered D epitopes and/or decreased antigen copies per red cell. Individuals carrying these variants may test antigen negative, weakly positive, or positive by serology, and may or may not be at risk of alloimmunisation after exposure. There have been recommendations to perform *RHD* genotyping of patients, pregnant women and females of childbearing potential with serological weak D phenotype, to guide prophylactic use of Rh immune globulin (RhIG), and better conserve D-negative blood products. The purpose of this study was to evaluate the performance of a set of empirical criteria to identify such patients.

Materials and methods. A two-method strategy of gel testing (GT) and tube testing (TT) was used for Rh typing of patients with no historical blood type in the present institution. A monoclonal-polyclonal blend anti-D was used for Rh typing by TT at immediate spin. Three empirical criteria were used to identify candidates for genotyping: C1: discrepancy between the two test methods and a GT reaction strength >2+ stronger than TT; C2: weak serological reaction, defined as reaction strength ≤2+ regardless of testing method if both GT and TT were performed or reaction strength ≤2+ if only GT was performed, or reaction strength ≤1+ if only TT was performed; C3: presence of anti-D in D-positive patients with no history of RhIG use in the preceding 3 months and in whom alloanti-D is suspected.

Results. Overall, 50 patients, ranging from newly born to 93 years old, were identified. Genomic testing confirmed D variants in 49/50 cases with a positive predictive value of 98%.

Discussion. This two-method strategy is a powerful screening tool for identifying candidates for *RHD* genotyping. This strategy meets the current requirements of two blood type determinations/two specimens in pre-transfusion testing while simultaneously identifying candidates for *RHD* genotyping with a minimal increase in work load and cost.

Keywords: RhD phenotyping, *RHD* genotyping, Rh immunoglobulin, weak D, partial D.

Introduction

Rh is the second most important clinically significant blood group system in transfusion medicine after the ABO system. Since the initial discovery of RhD[1], more than 50 antigens within the Rh system have been identified[2]. *RHD* and *RHCE* are closely linked homologous genes that encode these Rh group antigens[3]. More than 200 *RHD* variants have been recognised to date[4,5].

D variants are sorted into three major categories, weak D, partial D, and *DEL*, based on genotypes and potential for making alloanti-D. D variants can be typed positive, negative or weak. A serological weak D phenotype is defined as no or weak (≤2+) red blood cell reactivity to an anti-D reagent with non-antihuman globulin testing but moderate to strong agglutination with antihuman globulin testing[5-9]. Weak D variants express amino acid substitutions in the intramembranous and/or cytoplasmic regions of RhD which affect antigen expression and/or exposure on the red blood cell surface[7,11]. Of the more than 70 weak D variants, there are sufficient data showing lack of alloanti-D formation for the three most common D variants, weak D types 1, 2, and 3, to support not giving carriers prophylaxis[12,13], whereas this is not the case for certain other D variants[11,12,14-16].

Partial D is defined as incomplete expression of the multiple RhD epitopes on the red blood cell surface. Variants in this category include those with amino acid and exon substitutions in the extracellular region of the RhD protein which destroy one or more epitopes[11]. The strength of red cell reactivity to an

anti-D reagent depends on the epitope specificity of the reagent, which is the rationale behind partial D phenotyping using monoclonal anti-D reagent panels. It is possible for partial D recipients to be D-positive in serological testing yet still produce alloanti-D following transfusion of D-positive red blood cells[17-21], presumably by mounting a reaction against D epitopes that they lack.

A recent College of American Pathologists (CAP) Transfusion Medicine Resource Committee survey found that in the United States, most transfusion service laboratories do not use indirect antihuman globulin testing on potential blood product recipients and women of childbearing age in order to avoid detecting serological weak D individuals in this population and identifying them as D-negative[22]. For blood donors and neonates, however, the AABB standards require that laboratories test for serological weak D and interpret positive tests as D-positive in order to minimise exposure of D antigen-negative subjects to weak D-positive red blood cells via transfusion, and to administer Rho(D) immune globulin (RhIG) to D-negative mothers of weak D babies[23].

Based on the results of this survey and a systemic review of literature, a Work Group formed by the AABB and CAP, with scientific consultants knowledgeable in *RHD* genetics, developed the following recommendations aimed to change clinical management of serological weak D patients: (i) *RHD* genotyping should be performed when an inconsistency in RhD typing results and/or a serological weak D phenotype is identified; (ii) recipients with weak D type 1, 2, and 3 alleles should be reported and managed as D-positive individuals; (iii) women of childbearing age with weak D phenotypes who are not type 1, 2, or 3 should receive RhIG prophylaxis. The Work Group posited that by implementing these recommendations, RhIG use would be minimised and D-negative units better preserved without increasing the incidence of alloimmunisation to the D antigen[10]. The multi-organisational Joint Statement recently published on the AABB website also recommends *RHD* genotyping for pregnant women and other females of childbearing potential with a serological weak D phenotype[24].

It is of primary importance to establish practical criteria to identify candidates for *RHD* genotyping to implement these recommendations. Although the Work Group suggests using a discordant RhD typing result as a screening test[10], consistent criteria to identify discordant serological types for *RHD* genotyping are currently lacking. The purpose of the present study is to evaluate the performance of a set of empirical criteria to identify candidates for *RHD* genotyping.

Materials and methods

Institution and subjects

This study was conducted at a tertiary medical centre with approximately 15,000 red blood cell unit transfusions, 2,300 births, and 14,500 surgical operations annually. The main hospital does not serve paediatric patients; however, the Transfusion Service provides its services to a children's hospital with about 1,200 operations per year. In addition, annually, the Transfusion Service serves about 10,000 new patients with no prior ABO/Rh type results in the electronic record system. All new patients met inclusion criteria for this study.

For the retrospective phase of this study, all available *RHD* genotype reports on file since 2006 were reviewed to identify qualified cases that met at least one of the screening criteria for *RHD* genotyping (14 cases). Since January 2015, all cases that met one or more of the criteria (36 cases) were enrolled in the prospective phase of the present study, and the specimens were sent for *RHD* genotyping.

Serological RhD phenotyping

In order to comply with AABB Standards for Blood Banks and Transfusion Services[23], ABO/Rh typing is performed twice on the first specimen for all new patients. To comply with CAP requirements, a second specimen is required prior to transfusion for non-emergency patients. Prior to late 2011, all Rh testing was performed using a monoclonal-polyclonal blend anti-D (BioClone; Ortho Clinical Diagnostics, Raritan, NJ, USA) by tube testing (TT), with additional weak D testing (anti-globulin phase) as needed. In late 2011, gel testing ([GT]; Ortho ProVue; Ortho Clinical Diagnostics) was implemented as the primary method for ABO/Rh typing and antibody screening. Since June 2012, both methods (GT and TT) have been used for ABO/Rh typing on the first specimens from new patients except for neonates (see text below about neonatal testing). The first ABO/Rh typing is performed by GT, which is followed by a second ABO front typing and Rh typing (immediate spin phase only) by TT. Cases that met one or more of the criteria below were sent for *RHD* genotyping.

According to the manufacturer's instructions, EDTA plasma is required for GT. In our institution, all cord blood specimens are collected into red-top tubes without anticoagulant. In addition, heel-stick specimens are usually of small quantity (~0.3 mL on average) and not sufficient for GT by ProVue. Therefore, for neonates, only the TT method is used for both the initial and the second ABO/Rh typing, using the same specimen.

Screening criteria for *RHD* genotyping

A previous study showed that GT performs as well as TT in terms of serological RhD phenotyping[25]. Unpublished data from the present institution also suggest that red blood cells carrying D variants tend to react more strongly with GT than with TT. Based on this observation, the following empirical criteria were developed and used to identify candidates for *RHD* genotyping in the present study: (criterion 1: C1) discrepancy between the two testing methods: GT reaction strength at least 2+ stronger than TT; (criterion 2: C2) serological weak reaction defined as: agglutination observed (i.e. reactive), however reaction strength ≤2+ regardless of testing method if both GT and TT were performed, or reaction strength ≤2+ if only GT was performed, or reaction strength ≤1+ if only TT was performed, which is different from the Work Group recommendations; (criterion 3: C3) presence of anti-D in D-positive patients with no history of RhIG use in the preceding 3 months and in whom alloanti-D is suspected. For C2, the reaction strength cut-off was established at ≤2+ if only GT was performed because it was presumed that the TT reaction would not be stronger than the GT one, whereas the cut-off was set at ≤1+ if only TT was performed so if a second GT were to be performed with the same specimen, C2 would still be satisfied if the GT reaction strength were ≤2+, or C1 would be satisfied if the GT reaction strength were >2+. By definition, C2 is not met if only TT is performed and no agglutination is observed (i.e. non-reactive), since a negative TT by itself does not favour weak D/partial D over a true D-negative.

***RHD* genotyping**

All *RHD* genotyping was performed by a reference molecular laboratory. Genomic DNA was isolated from peripheral blood using standard methodology. *RHD* genotyping included testing by RHD BeadChip (Immucor, Norcross, GA, USA)[26], multiplex polymerase chain reaction (PCR) for C, c, *RHD* exon 4, exon 7 and *RHD* pseudogene[27], hybrid Rhesus Box PCR[28] and PCR-restriction fragment length polymorphism with PstI for *RHD* deletion[29]. PCR-restriction fragment length polymorphism for *RHD* c.667, c.697, c.1136 and sequence-specific primer PCR for c.455 were performed as described previously[30] with restriction enzymes from New England Biolabs (Ipswich, MA, USA). Sanger sequencing of *RHD* exons was performed if necessary to assign the alleles and to resolve indeterminate calls generated by RHD BeadChip (P14, P20, P31, P33, P39) using primers as described[30].

When no variants were detected by targeted genomic testing and a D variant was strongly suspected, total RNA was isolated from red cells using an RNeasy Minikit (Qiagen, Carlsbad, CA, USA), cDNA was prepared using Superscript III (ThermoFisher, Waltham, MA, USA), *RHD* cDNA was amplified and Sanger sequencing was performed (P40).

Results

A total of 50 cases (32 women and 18 men), ranging from a neonate (P35) to a 93-year old (mean ± standard deviation [SD]: 40±19 years) were identified. Fourteen of these were identified through retrospective review, and the remaining 36 cases prospectively (Table I). Twenty-seven cases were African-Americans, 16 were Hispanics, six were Caucasians, and one was of unknown race/ethnicity. This race/ethnicity constitution was not statistically significantly different from the estimated race/ethnicity constitution of the patient population at the institution (based on 2015 data) (χ^2=10.3, degrees of freedom [DF]=5, p=0.066; Table II). Twenty-three cases met C1 only, nine met C2 only, three met C3 only, 13 met both C1 and C2, one met both C1 and C3, and one met all three criteria. Genotyping confirmed D variants in 49 cases (10 [20%] with weak D and 39 [78%] with partial D), with a positive predictive value of 98% (49/50) (Table I).

Among the 39 partial D subjects, 20 (51.3%) met C1 only, four (10.3%) met C2 only, two (5.1%) met C3 only, 11 (28.2%) met both C1 and C2, one (2.6%) met both C1 and C3, and one (2.6%) met all three criteria. GT reactivity among those with partial D variants ranged from 1+ to 4+, whereas TT reactivity was mostly 1+ or non-reactive, with one 2+ (P1, who met C1) and one 4+ only (P38, who met C3). The greatest discrepancy in reactivity seen in this group of patients was 4+ in GT and non-reactive (-) in TT (P5). Within the ten weak D subjects, two (20%) met C1 only, five (50%) met C2 only, none met C3 only, three (30%) met C1 and C2, none met C1 and C3, and none met all three criteria. GT reactivity among these subjects ranged from 1+ to 3+, whereas TT reactivity was mostly 1+ or non-reactive. The greatest discrepancy in reactivity seen in this group of patients was a case that was 3+ in GT and non-reactive in TT. There was a statistically significant difference between partial D and weak D cases in percentage of criteria met (χ^2=18.3, DF=5, p=0.003; Table III). Reaction strength discrepancy (C1) was more likely to be observed among partial D subjects (84.6% in partial D *vs* 50% in weak D), whereas serological weak D phenotype (C2) was more prevalent among weak D subjects (41.0% in partial D *vs* 80% in weak D), as the nomenclature of weak D would suggest (Tables I and III).

Strong reactivity

Table I shows that 25 (P1-P21, P33, P39, P41, P42) out of 49 D variant cases demonstrated strong reactivity (3+ or 4+) in GT, but weak reactivity in TT except for

Table I - Patients' characteristics.

Patients	Age (years)	Gender	Race/ ethnicity	GT	TT	Reason for genotyping	Rh phenotype	*RHD* genotype
P1	52	F	B	4+	2+	C1	Partial D	*RHD*DAU/RHD*pseudogene*
P2	31	F	B	4+	1+	C1	Partial D	*RHD* weak partial D 4.0* (hemizygous or homozygous)
P3	55	F	B	4+	1+	C1	Partial D	*RHD*DAU5 / RHD*pseudogene (Ψ)*
P4	27	F	H	4+	1+	C1	Partial D	*RHD*DAU5* (hemizygous or homozygous)
P5	52	M	B	4+	0	C1	Partial D	*RHD*weak partial D 4.0 / RHD*01N.01*
P6	24	F	B	3+	1+	C1	Partial D	*RHD*DAU4* (hemizygous or homozygous)
P7	23	F	B	3+	1+	C1	Partial D	*RHD*DAU5* (hemizygous or homozygous)
P8	22	M	B	3+	1+	C1	Partial D	*RHD*DAU5 / RHD*DV type 1*
P9	33	M	B	3+	1+	C1	Partial D	*RHD*DAU5 / RHD*ψ*
P10	57	F	B	3+	1+	C1	Partial D	*RHD*pseudogene / RHD*weak partial D 4.0*
P11	49	M	B	3+	1+	C1	Partial D	*RHD*weak partial D 4.0 / RHD*DIIIa-CE(4-7)-D*
P12	25	F	H	3+	1+	C1	Partial D	*RHD*DAR* (hemizygous or homozygous)
P13	50	M	H	3+	1+	C1	Partial D	*RHD*DAR* (hemizygous or homozygous)
P14	49	F	H	3+	1+	C1	Partial D	*RHD*DOL1* or *RHD*DOL2* (hemizygous or homozygous)
P15	59	F	H	3+	1+	C1	Partial D	*RHD*weak partial D 4.0* (hemizygous or homozygous)
P16	43	M	U	3+	1+	C1	Partial D	*RHD*DAU5* (hemizygous or homozygous)
P17	42	F	B	3+	0	C1	Partial D	*RHD*DAR* (hemizygous or homozygous)
P18	19	M	B	3+	0	C1	Partial D	*RHD*DAU5*
P19	63	M	H	3+	0	C1	Partial D	*RHD*DAR / RHD*01N.01*
P20	23	F	H	3+	0	C1	Partial D	*RHD*DAU4 / RHD*01N.01*
P21	42	F	W	3+	0	C1	Partial D	*RHD*weak D Type 4.1 / RHD*01N.01*
P22	37	F	B	2+	0	C1, C2	Partial D	*RHD*DIIIa-CE(4-7)-D / RHD*DAR*
P23	85	M	B	2+	0	C1, C2	Partial D	*RHD*DAR*
P24	21	F	B	2+	0	C1, C2	Partial D	*RHD*DAR* (hemizygous or homozygous)
P25	19	F	B	2+	0	C1, C2	Partial D	*RHD*DAR / RHD*DIIIa CE(4-7)*
P26	64	M	B	2+	0	C1, C2	Partial D	*RHD*DAR1* (hemizygous or homozygous)
P27	60	M	B	2+	0	C1, C2	Partial D	*RHD*DAU5* (hemizygous or homozygous)
P28	33	F	B	2+	0	C1, C2	Partial D	*RHD*DIIIa-CE(4-7)-D / RHD*DAR*
P29	35	F	B	2+	0	C1, C2	Partial D	*RHD*IIIa/RHD*weak partial D 4.0* or *RHDIIIa-CE(4-7)-D/RHD*weak partial D 4.0*
P30	28	F	H	2+	0	C1, C2	Partial D	*RHD*DAR1* (hemizygous or homozygous)
P31	24	F	H	2+	0	C1, C2	Partial D	*RHD*DSC1* (hemizygous or homozygous)
P32	42	F	B	2+	0	C1, C2, C3	Partial D	*RHD*DAR* (hemizygous or homozygous)
P33	62	M	H	4+	1+	C1, C3	Partial D	*RHD*DSC1* or *RHD*DFV*
P34	25	M	B	NP	1+	C2	Partial D	*RHD*DAU5* (hemizygous or homozygous)
P35	NB	F	B	NP	1+	C2	Partial D	*RHD*DIII*weak partial D 4.0*
P36	47	M	B	2+	1+	C2	Partial D	*RHD*DAR* (hemizygous or homozygous)
P37	29	F	B	1+	0	C2	Partial D	*RHD*DAU5 / RHD*DIIIa-CE(4-7)-D*
P38	22	F	H	NP	4+	C3	Partial D	*RHD*DIVa / RHD*01N.01*
P39	29	F	H	3+	NP	C3	Partial D	*RHD*DIVa / RHD*01N.01*
P40	21	F	H	1+	0	C2	Weak D	*RHD*weak D type 61*

Continued on next page

Table I - Patients' characteristics *(continued from previous page)*.

Patients	Age (years)	Gender	Race/ ethnicity	GT	TT	Reason for genotyping	Rh phenotype	*RHD* genotype
P41	53	F	H	3+	1+	C1	Weak D	*RHD*weak D type 3* (hemizygous or homozygous)
P42	40	F	B	3+	0	C1	Weak D	*RHD*weak D type 1*
P43	54	M	H	2+	0	C1, C2	Weak D	*RHD*weak D type 2* (hemizygous or homozygous)
P44	64	F	W	2+	0	C1, C2	Weak D	*RHD*weak D type 1*
P45	27	F	W	2+	0	C1, C2	Weak D	*RHD*weak D type 2* (hemizygous or homozygous)
P46	26	F	H	NP	1+	C2	Weak D	*RHD*weak D type 3 / RHD*01N.01*
P47	57	M	W	2+	NP	C2	Weak D	*RHD*Weak D type 2 / RHD*01N.01*
P48	18	F	B	1+	0	C2	Weak D	*RHD*weak D type 2 / RHD*01N.01*
P49	55	M	W	1+	0	C2	Weak D	*RHD*weak D type 2* (hemizygous or homozygous)
P50	93	M	W	4+	4+	C3	D-positive	*RHD*weak D type 3 / RHD*01*

F: female; M: male; GT: gel testing; TT: tube testing; A: Asian/Pacific Islander; B: Africa-American/Black; H: Hispanic; W: White; U: unknown; NB: newborn; NP: not performed; 0: no agglutination observed.

Table II - Race/ethnicity of the 50 cases in the present study.

Race/ ethnicity	Number of cases who met the criteria	Percentage of cases who met the criteria	Percentage in the patient population of the present institution (2015 data)	Statistics
A	0	0.00	1.40	
B	27	54.00	39.72	
H	16	32.00	25.16	χ^2=10.3 DF=5 p=0.066
W	6	12.00	25.03	
U	1	2.00	4.85	
O	0	0.00	3.84	
Total	50	100	100	

A: Asian/Pacific Islander; B: Africa-American/Black; H: Hispanic; W: White; U: unknown; O: others; DF: degrees of freedom.

Table III - Criteria (C1-C3) met by the two different categories of D variant carriers.

	Partial D	Weak D	Statistics
C1	51.28	20.00	
C2	10.26	50.00	
C3	5.13	0.00	χ^2=18.3 DF=5 p=0.003
C1, C2	28.21	30.00	
C1, C3	2.56	0.00	
C1, C2, C3	2.56	0.00	

DF: degrees of freedom.

P39 (TT not performed). These cases would have been categorised as D positive if only GT had been used for typing, unless an allo-anti-D had been identified. Partial D variants were identified in 23 of these cases (23/25, 92%), whereas weak D variants were identified in two cases (2/25, 8%; P41, P42) only (Table I). Twenty-three of these cases (23/25, 92%; P1-P21, P41, P42) were identified because two methods were used and C1 was met.

Negative tube testing results

In the present study, 25 cases (P5, P17-P32, P37, P40, P42-P45, P48, P49) out of 50 (50%) were non-reactive with TT, and would have been missed and managed as D negative, had GT not been performed as a second method. Seven of these cases (7/24, 29%) were weak D. Among these weak D cases, six cases (6/7, 85.71%; P42-P45, P48, P49) were type 1, 2 or 3, and the remaining one (P40) was weak D type 61 (Table I).

Cases with anti-D

RHD genotyping was performed in five cases (P32, P33, P38, P39, P50) because C3 was met (alone or in combination with other criteria), i.e., anti-D was present in a D-positive patient with no history of RhIG use in the preceding 3 months in whom allo-anti-D was suspected (Table IV). The auto control was weakly positive (1+) by GT in two cases (P32, P50). The direct antiglobulin test (DAT) by TT was negative and, therefore, no eluate or autoadsorption was performed on these cases according to laboratory procedures. However, we recognise that for a case of positive auto control by GT, the DAT should be

Table IV - Characteristics of the patients expressing anti-D.

Patient	Age (years)/ gender	Interpretation	Alloanti-D propensity	Obstetric history	Transfusion within preceding 3 months	RhIG within preceding 3 months	Anti-D status	Autocontrol	DAT by TT	Eluate/auto-adsorption
P32	42/F	Partial D	DAR previously reported[7]	G3/P2/A1	Yes, 5 weeks ago	No	Present	GT: 1+ PEG: 0	0	NP/NP
P33	62/M	Partial D	DFV previously reported[7]	NA	No	No	Present	0	0	NP/NP
P38	22/F	Partial D	DIVa previously reported[7]	G4/P2/A2	Yes	No	Present	0	0	NP/NP
P39	29/F	Partial D	DIVa previously reported[7]	G8/P4/A3	No	No	Present	0	0	NP/NP
P50	93/M	D-positive	NA	NA	No	No	Present	GT: 1+	0	NP/NP

DAT: direct antiglobulin test; TT: tube testing; GT: gel testing; NA: not applicable; NP: not performed; 0: not reactive; G/P/A: gravida/para/abortus; PEG: polyethylene glycol.

performed in GT and should also be positive. However, TT is the only method validated in our laboratory for DAT; GT is not validated for DAT in our laboratory and DAT by GT was not, therefore, performed. Genotyping categorised four of the five cases as partial D (P32, P33, P38, P39) and one as D-positive (P50). Of the four partial D subjects who demonstrated alloanti-D antibodies, three (P32, P38, P39) were multiparous females (P38 also had a recent history of transfusion with Rh-positive red cells at the present institution) and one was a male (P33) with no known transfusion history. P38 tested strongly D positive (4+) with TT (GT not performed), whereas P39 tested moderately D positive (3+) with GT (TT not performed). Three cases (P33, P38, P39) tested negative in the auto control, which is typical for partial D subjects serologically demonstrating alloanti-D. P32 tested weakly positive for the auto control (1+) with GT. Although an autoanti-D component could contribute to the weak anti-D reactivity in P32, the possible role of alloanti-D cannot be completely ruled out, given the partial D phenotype in this patient. The D variants in these subjects, *DAR* (P32), *DFV* (P33), and *DIVa* (P38 and P39), have been associated with alloanti-D production[8]. The fifth subject in this group (P50) is a 93-year old male with no known transfusion history. The patient's red blood cells demonstrated strong serological reactivity (4+) with both GT and TT, and tested weakly positive for auto control (1+) with GT. The DAT by TT was negative and, therefore, elution was not performed according to laboratory procedures. This patient's genotype was a normal D allele and a weak D type 3 allele such that his phenotype is D-positive.

Discussion

Both the AABB[23] and CAP[30] require two specimens be tested for non-emergency pre-transfusion testing, whereas to date, no regulations require the use of two different methods for RhD typing. In addition, all current RhD serology typing reagents/methods on the US market are approved for independent clinical use, and hence confirmation using a second reagent is not required. Most hospitals do not, therefore, mandate RhD typing with two testing methods. In most studies published to date[32,33], *RHD* genotyping was performed because of either weak reactivity or Rh type discrepancy noticed incidentally in practice such as historical type *vs* current type, donor type *vs* recipient type, or between facilities. In our hospital, since June 2012, ABO front typing and Rh typing have been performed twice by two different methods (GT and TT) on the first specimen from all patients without historical blood type information. In addition, a second specimen has been required for ABO/Rh group confirmation for non-emergency transfusion. In the present study, we report on the use of these two methods for Rh typing and the discrepancy in reactivity between them, as a complementary criterion (C1) in addition to weak reactivity (C2) and the presence of anti-D in D-positive subjects (C3), to screen individuals for *RHD* genotyping.

As early as 1995, a strategy of using two monoclonal antibodies to identify DVI variants was proposed by Wagner and Colleagues[33]. Subsequently, other researchers also noted that a two-monoclonal anti-D strategy could be useful in identifying D variants[34]. There are some similarities and differences between a two-monoclonal antibody strategy and a two-method strategy. The basic principle of both strategies is the same, i.e., using discordance between two different test results to identify samples that may express a D variant. A two-monoclonal anti-D strategy is efficient at identifying specific D variants of interest, for example DVI, which is common in individuals of European

descent and clinically important due to the risk of alloimmunisation. Our approach, which is not limited to the use of monoclonal anti-D reagents, was designed to detect more variants, which would be expected in a multi-ethnic population. In addition, both anti-D reagents used in the two testing methods (TT and GT) in this study are approved by the US Food and Drug Administration for independent use and both can detect normal D and most variant types. Our approach uses both TT and GT. TT is performed with a monoclonal-polyclonal blend anti-D and GT is performed using a monoclonal human IgM anti-D secreted by a mouse/human hybridoma. The differences in both test mechanisms and antibodies used are contributors to the sensitivity in detecting discrepancies between the two methods.

The criteria are highly predictive for *RHD* variants

Among all the 50 patients who met at least one of the criteria, D variants were detected by targeted genomic testing in 48 cases (10 [20%] with weak D and 38 [78%] with partial D) and by cDNA sequencing in one case (P40, weak D) with a positive predictive value of 98% (49/50) (Table I).

When *RHD* genotyping was limited to targeted testing, which included use of a commercial array (RHD BeadChip) and several laboratory-developed tests for variants not interrogated on the array, D variants were identified in 48 of 50 patients. When the patient with no variants identified by targeted testing (P40) was tested using the higher resolution cDNA analysis, a D variant (weak D type 61) was detected. This case illustrates that when targeted genomic testing fails to detect a variant, the negative test result does not fully exclude the possibility of a D variant. The commercial *RHD* genotyping products test for many variants commonly found in individuals of Caucasian and African ancestry. Given the Hispanic ethnicity of this female patient and her GT 1+, TT negative serology reactivity, it was strongly suspected that she carried an RhD variant that was not detectable by the targeted genomic testing used. cDNA analysis is useful to detect other variants in such scenarios.

The two-method strategy is more sensitive

The criteria described here are more sensitive at identifying subjects likely to express weak or partial D variants than weak reactivity of <2+ (C2) using only one method such as GT or TT. As mentioned above, the cases with strong reactivity (≥3+) by GT, and those cases negative by TT would not have been identified without using a second method (Table I).

Some partial D individuals in the present study would have been typed as D-positive if only one method had been used, which is the current common practice in many facilities. Specimens from 23 partial D patients (P1-P21, P33, P39) reacted strongly (3+ or 4+) with GT. Had it not been for the reactivity discrepancy identified by the two-method strategy, these partial D patients would have been managed as D-positive patients putting them at risk of developing allo-anti-D antibodies after exposure to D-positive blood.

Similarly, a total of ten weak D cases (weak D type 1, 2 or 3) were identified by targeted genotyping in the present study. Of these ten cases, six (P42-P45, P48, P49) were D-negative by TT. These cases would have been improperly categorised had the GT not been performed. However, if all specimens with negative TT were sent for *RHD* genotyping, the yield (i.e. specificity) of detecting D variants would be low, since negative TT by itself is not able to differentiate true D-negative from D variant cases. All these six weak D patients (P42-P45, P48, P49) were either weak D type 1 or type 2 patients (Table I), and could be managed safely as D-positive recipients. RhIG prophylaxis and Rh-negative red blood cell products are not required (since the chance of producing alloanti-D is low[12,13]), which helps to save D-negative blood products and RhIG resources.

These results demonstrate that the two-method reactivity discrepancy criterion (C1) can help to identify candidates for *RHD* genotyping. This is not only because individuals with weak reactivity are more likely to be identified by two methods rather than one, but more importantly because the discrepancy in reaction strength can be appreciated only by using two methods. Since both the AABB and CAP require two blood typing determinations in pre-transfusion testing, and performing two blood typing tests by two different methods can be done with minimal additional labour or expense, and useful information is obtained from possible discrepancies in reactivity between the two methods, this strategy should be considered by hospital transfusion services for testing of all new patients.

Reactivity discrepancy was more likely to be seen with partial D than weak D specimens (Results and Table III). This is not surprising, as partial D variants have altered or missing RhD epitope(s) such that use of different reagents and different methods may give disparate results. If a particular epitope detectable by the anti-D reagent used is lacking in a partial D specimen, the reactivity observed with this anti-D reagent is expected to be weak or negative. However, the epitope detectable by another anti-D reagent may be present in this partial D specimen, and the reactivity would be strong if the other anti-D reagent is used. Thus, the discrepancy between GT and TT in reactivity is a useful screening method to identify specimens requiring *RHD* genotyping, especially for partial D variants.

Criterion 3 (presence of anti-D in a presumed D-positive patient) is complementary to criterion 1 (discrepancy) and criterion 2 (weak reaction)

Five cases (P32, P33, P38, P39, P50) met C3, all of whom were serologically D-positive by history and/or typing in the present institution and/or other facility, and demonstrated anti-D antibodies with no concurrent non-specific warm autoantibodies. Genotyping identified four of these five as partial D (P32, P33, P38, P39) and one as D-positive (P50). Three cases (P38, P39, P50) met C3 only, and two cases (P32, P33, both partial D) met C3 and at least one other criterion (Table IV).

Case P38 (partial D, *RHD*DIVa*) was strongly D-positive (4+) by TT (GT not performed). This case would not have been considered a candidate for *RHD* genotyping without the identification of anti-D. P39 (partial D, *RHD*DIVa*) tested moderately D-positive (3+) with GT (TT not performed). Case P50 demonstrated strong serological reactivity (4+) with both GT and TT. This case would not have been considered as a candidate for *RHD* genotyping, had anti-D not been identified. *RHD* genotyping showed that this patient carries an apparently conventional D allele suggesting that the anti-D in this patient is likely to be an autoantibody.

RHD genotyping can identify variants in patients in whom anti-D is identified in serologically D-positive individuals. Both P32 (female, partial D) and P50 (male, D positive) tested weakly positive for auto control (1+) with GT. Patient P32 typed D-negative and received RhIG during her pregnancy at an outside hospital about 10 years before the current specimen was taken. However, 5 weeks prior to the date of the current specimen, she typed D-positive at another outside hospital and received two units of D-positive red blood cells. Our workup showed Rh type 2+ with GT, negative with TT, positive for anti-D antibody with positive auto control (1+). The DAT was negative and eluate tests were not performed. Auto-adsorption was not performed because of the recent transfusion. The patient had not received RhIG in the 3 months prior to the current specimen being taken. Genotyping detected *RHD*DAR* (hemizygous or homozygous), which has been reported to produce alloanti-D antibody following exposure to D-positive blood. This patient's anti-D is, therefore, probably an alloantibody

Patient P50 was strongly positive (4+) with both GT and TT. He had no recent history of transfusion or RhIG use within the 3 months prior to the current specimen being taken. Auto-adsorption was not performed. *RHD* genotyping confirmed the presence of a normal D allele and D-positive phenotype.

P33, a 62-year old Hispanic man, was seen initially in mid-2015 for pre-transplant evaluation for end-stage renal disease and had a past medical history of diabetes mellitus, hypertension, and childhood asthma. He did not speak English, and an interpreter was present for the entire encounter. He was admitted to an outside hospital 3 months previously and did not recall any history of blood transfusion or RhIG administration. He was strongly D-positive (4+) with GT and weakly D-positive (1+) with TT. No agglutination was observed with the auto control, suggesting that the anti-D antibodies were the result of allo-immunisation, most probably due to a previous, remote exposure to a D-positive blood product.

More D variant cases, especially partial D, identified in the current study

In the present study, the D variants identified using our screening criteria consisted of more partial D cases (39/50; 78%) than weak D cases (10/50; 20%). These results are different from published data[26,27]. In a study by Van Sandt *et al*.[27] weak D type 1, 2, or 3 was identified in 424/628 (67.5%) cases, type 4.0/4.1/4.3, 4.2, 5, 11, 15, or 17 was identified in 22/628 (3.5%) cases, and partial D variants were identified in 49/628 (7.8%) cases (mainly DVI types, n=27). In a study published by Haspel *et al*.[26], weak D type 1, 2 or 3 was identified in 27/36 cases (75%), whereas partial D variants were identified in only 9/36 cases (25%). It is known that types of the D variants and their frequencies in the population vary with race/ethnicity. A difference in race/ethnicity between this and prior studies may contribute to, but is not likely to be the only reason behind, the significant difference in constitution of partial D between our study (78%) and other studies (7.8-25%). Using two typing methods and the discrepancy criterion (C1) for screening is probably a prominent contributor to the higher partial D variant constitution observed in the present study. We hypothesise that the difference in sensitivity to D variants between the two typing methods is the key contributor to the difference in the constitutions of D variants between our study and other published studies.

Conclusions

The three-criteria approach proposed in the present study is a useful screening tool for identifying candidates for *RHD* genotyping, with a high positive predictive value of 98%. This approach is more sensitive than using a single method and is more sensitive for partial D. Incorporating the two-method discrepancy strategy into the current AABB and CAP requirements for two blood type determinations/specimens in pre-transfusion testing will help to identify more candidates for *RHD* genotyping with a minimal increase in work load and cost.

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Authorship contributions

XL, IJ, MG, SL and YL designed the study. IJ and KSM collected the data. XL performed the data analysis. XL, MAK, AC, SN and YL analysed and interpreted the data. XL and YL wrote the manuscript. MAK wrote the genotyping section. All Authors critically reviewed, edited and approved the manuscript.

The Authors declare no conflicts of interest.

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