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RHD Genotyping to Resolve Weak and Discrepant RHD Phenotypes: The "Serenissima" Experience

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Authors' contributions

This work was carried out in collaboration between both authors. The authors both contributed equally to the collection of data and the drafting and revision of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Background: A considerable number of RHD alleles responsible for weak and partial D phenotypes have been identified. Serological determination of these phenotypes is often doubtful and makes genetic analysis of RHD gene highly desirable in transfusion recipients and pregnant women.
Aim: We report the experience of Mestre Blood Bank in analysis of the RHD gene in six years from 2018 to 2023.
Methods: Subjects for RHD gene analysis were selected for presence of a serological weak D phenotype, defined as reactivity of RBCs with an anti-D reagent giving no or weak (≤2+) score in initial testing but agglutinating moderately or strongly with anti human globulin (AHG). These samples were selected for genotyping using the microarray-based method Bead-Chip supplied by Werfen.

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Results: From 2018 to 2023, we selected, for RHD gene analysis, 555 subject with D weak phenotype; 86 subjects (15.5%) were D positive and 56 (10.1%) were D negative, without variant, in 413 subjects a D weak or a D variant was observed.
Discussion: Many serological weak D phenotypes are associated to RHD gene mutations leading to one or more amino acids substitutions in the RhD protein predicted to be within or below the RBC membrane, causing decreased antigen expression on the red cell surface. Prevalence of serological weak D phenotypes varies by race and ethnicity. Serological weak D phenotypes are the most common D variants detected in Caucasians (0.2%-1.0%). The majority, as in our series, are associated with weak D type 1, 2 or 3. Our data confirmed a high prevalence of weak D type 1 and type 2, but we observed a high prevalence of type 11 and 15 and of the uncommon type 18 too. The most common partial D phenotypes in Europe are DNB, DVI, and DVII. Our data confirmed a high prevalence of D partial type VI. Studies indicate that D partial transfusion recipients are at risk of forming alloanti-D when exposed to conventional RhD-positive blood units.

Keywords: D variant; D weak; genotypes; RHD.

1. INTRODUCTION

The Rh blood group system consists of 56 antigens carried on two proteins (RhD and RhCE) each consisting of 417 amino acids. Combinations (hybrids) between the two genes are not uncommon. The proteins consist of 12 membrane-spanning domains. The inheritance of Rh system antigens is determined by a complex of 2 closely associated genes located on chromosome 1: a *RHD* gene which encodes the D protein which confers D antigenic specificity; in D negative Caucasian individuals the *RHD* gene is usually deleted while in other populations the D negative phenotype is associated with an inactive, mutated or partially active *RHD* gene. A *RHCE* gene which codes for proteins that confer the antigenic specificities C,c,E,e: the alleles are *RHC*e, *RHCE*, *RHcE* and *RHce.* [1-3] The RHD antigen is the most important and immunogenic antigen of the Rh blood group system. Correct identification of RhD antigen is of great clinical significance to prevent allo-immunization leading to post transfusion haemolytic reactions and to foetal and neonatal haemolytic disease [4,5]. Usually, serotyping is the standard method to study RhD antigen, and serological studies have distinguished three broad categories of D variants, namely, weak D, partial D and DEL, from wild-type or conventional D. Usually a D positive subject expresses a strong positive reaction (3/4+) in serological tests, conversely a serological weak D phenotype is defined as reactivity of RBCs with an anti-D reagent giving no or weak (≤2+) reactivity in initial testing but agglutinating moderately or strongly with antihuman globulin (AHG). Partial D phenotypes are associated with amino acid substitutions in the RhD protein on the RBC surface and lack of D epitopes. DEL phenotypes present by conventional blood typing as RhD negative and are not detected serologically unless adsorption and elution studies are performed [6-8]. Genotyping by molecular techniques is a complementary tool to overcome these limitations [9,10].

In this paper we report a six years' experience about the utility of a genotyping based approach to resolve weak and discrepant D serotyping in a large Urban Tertiary Care Hospital in North-East Italy.

2. MATERIALS AND METHODS

2.1 Serological Assay

Routine RhD serotyping was performed, in EDTA whole blood, using a commercial gel card assay: DG Gel 8 ABO/Rh (2D) supplied by Grifols Italy (MI). Assays were performed following the manufacturer's instructions. The principle of the test is based on the gel technique described by Yves Lapierre [11]. The DG Gel 8 cards are composed of eight microtubes. Each microtube is made of a chamber, also known as incubation chamber, at the top of a long and narrow microtube, referred to as the column. Buffered gel solution containing specific antibody (anti-A, anti-B, anti-AB, anti-DVI- or anti-DVI+) has been prefilled into the microtube of the plastic card. Fig. 1 reported the gel card structure: Microtube A: monoclonal antibody anti-A. Mixture of IgM and IgG antibodies of murine origin, clones 16243G2 and 16247E6. Microtube B: monoclonal antibody anti-B. IgM antibody of murine origin, clone 9621A8. This reagent does not react with acquired B cells. Microtube AB: monoclonal antibodies anti-AB. Mixture of IgM antibodies of murine origin, anti-A(B) clone

ES15, anti-A clone LA-2, and anti-B clone LB-2. Microtube DVI: monoclonal antibody anti-D. IgM antibody of human origin, clone P3x61. This reagent does not detect partial DVI. Microtube DVI+: monoclonal antibody anti-D. Mixture of IgM antibodies of human origin, clones P3x61 and ESD1M. This reagent detects partial DVI. Microtube Ctl.: buffered solution without antibodies (control microtube). Microtubes N: buffered solution without antibodies for the ABO reverse group test. Agglutination occurs when the red blood cell antigens react with the corresponding antibodies, present in the gel solution or in the serum or plasma sample (in the case of reverse grouping test). The gel column acts as a filter that traps agglutinated red blood cells as they pass through the gel column during the centrifugation of the card. The gel column separates agglutinated red blood cells from non-agglutinated red blood cells based on size [11]. Any agglutinated red blood cells are captured at the top of or along the gel column, and non-agglutinated red blood cells reach the bottom of the microtube forming a pellet. Figure 2 reported the semi quantitative score system for agglutination interpretation [12,13]. Samples resulting D negative are tested for weak D using two stage assay with antiglobulin reagent. In case of positivity, a direct antiglobulin test (DAT) is performed to exclude interference from autoantibodies.

Assays were performed following the manufacturer instructions using three Grifols Erytra Eflexis fully automated analysers.

2.2 Genotyping Assay

Routine genotyping was performed, in EDTA whole blood, using the commercial Immucor BioArray HEA BeadChip kit supplied by Werfen. Assays were performed following the manufacturer's instructions. This assay allow the molecular characterization of allelic variants that predict erythrocyte antigen phenotypes in the Rh (C [RH2], c [RH4], E [RH3], e [RH5], V [RH10], VS [RH20]), Kell (K [KEL1], k [KEL2], Kpa [KEL3], Kpb [KEL4], Jsa [KEL6], Jsb [KEL7]), Duffy (Fya [FY1], Fyb [FY2], GATA [FY-2], Fyx [FY2W]), Kidd (Jka [JK1], Jkb [JK2]), MNS (M [MNS1], N [MNS2], S [MNS3], s [MNS4], Uvar [MNS-3,5W], Uneg [MNS-3,-4,-5]), Lutheran (Lua [LU1], Lub [LU2]), Dombrock (Doa [DO1], Dob [DO2], Hy [DO4], Joa [DO5]), Landsteiner-Wiener (LWa [LW5], LWb [LW7]), Diego (Dia [DI1], Dib [DI2]), Colton (Coa [CO1],Cob [CO2]), and Scianna (Sc1[SC1], Sc2 [SC2]) blood group systems in human genomic DNA. The procedure starts with extraction of DNA from ethylenediaminetetraacetic acid (EDTA)–collected whole blood, using the QIAcube (Qiagen, Inc). The DNA segments of interest (which contain the sequence variations that are the basis for the phenotypic variations) are amplified by a multiplexed polymerase chain reaction (PCR) using a Veriti thermal cycler supplied by applied Biosystem. The resulting PCR product is treated to remove residual primers and deoxynucleotide triphosphates and to generate single-stranded DNA. The amplified, single-stranded DNA then anneals with oligonucleotide allele-specific probes that are attached to microscopic beads (each approximately 3.2 microns in diameter). These beads have been dispersed onto a chip containing approximately 4000 wells; each well is large enough to accommodate only 1 bead. The bead chips come in 96-chip formats. The beads have a characteristic fluorescent signature specific for each allele-specific probe. The location of the beads (with their attached allele-specific probes) on the chip is documented at the manufacturing site. When the amplified DNA is perfectly matched to the probe, it undergoes elongation and incorporates a fluorescently labelled nucleotide (a process called elongation mediated multiplexed analysis of polymorphisms). Only perfectly matched DNA segments will elongate and incorporate the fluorescent label. The bead fluorescence profile is captured by a Nikon AIS400C fluorescence microscope and analyzed by the BioArray Solutions Information System (BASIS; Immunocor, Inc.), which translates the fluorescence signal profile into genotype determination and phenotype prediction [14,15].

Our Laboratory is accredited according to Italian National criteria and is also accredited by the European Federation of Immunogenetics (EFI). Therefore all analytical methods used must be approved by the European Community for use as in vitro diagnostics (CE-IVD markings) and before being adopted in routine they must be subjected to validation according to the standards of the Italian Society of Transfusion Medicine and Immunohematology (SIMTI) [15,16].

Each analytical session is validated through evaluation of internal quality controls (IQC) and the performance of the methods is evaluated through participation in appropriate externalquality control programs (EQCP).

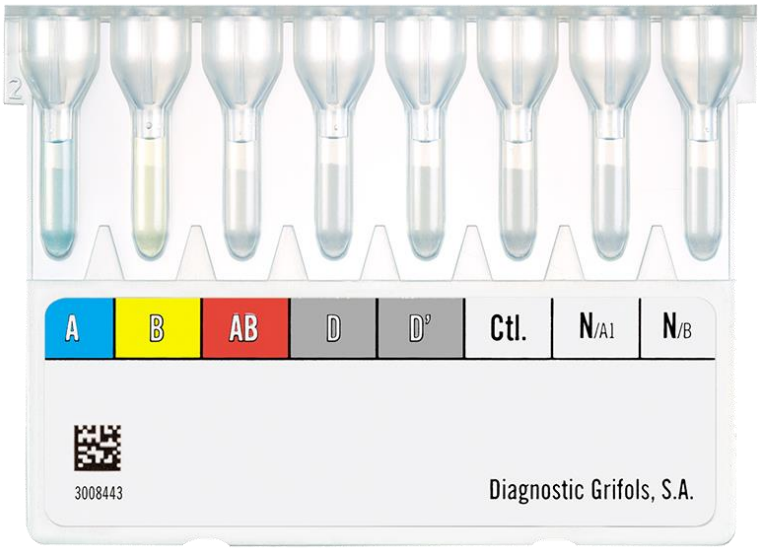

Fig. 1. Description of the Grifols DG Gel 8 ABO/Rh (2D) gel card

Fig. 2. Semiquantitative scoring system for agglutination interpretation

2.3 Samples Selection

In our Laboratory subjects for RHD gene analysis were selected based on the presence of one of the following criteria:

- Observation of a serological weak D phenotype, defined as reactivity of RBCs with an anti-D reagent giving no or weak (≤2+) score in initial testing but agglutinating moderately or strongly with antihuman globulin.
- Samples D negative with positive assay for D weak and positive direct antiglobulin test (DAT).
- Samples showing discordant reactivity in anti-DVI- / anti-DVI+ microtube [17].

3. RESULTS

From January 2018 to December 2023, considering samples selected as previously reported, in our Laboratory were performed RHD gene analysis in 555 subjects with age from 1 to 79 years, 382 (68.8%) were females and 173 (31.2) were males. As regards the origin of the genotyped subjects, the great majority (267, 48.1%) from the maternal and child department, 131 (23.6%) from the oncology and hematology department, 61 (11.0%) were outpatients, 24 (4.3%) were blood donors, 72 (13%) came from other hospital departments. Of the 555 subjects only 31 (5.6%) were of non-Caucasian ethnicity.

Table 1. RHD genotyping results

RHD Genotype	Case N° (%)	RHD Genotype	Case N° (%)
D weak type 1	227 (40.8%)	D partial type III	3 (0.5%)
D weak type 2	46 (8.3%)	D partial type IV	1 (0.2%)
D weak type 4	4 (0.7%)	D partial type V	4 (0.7%)
D weak type 11	47 (8.4%)	D partial type VI	27 (4.9%)
D weak type 15	34 (6.1%)	DAR	1 (0.2%)
D weak type 18	4 (0.7%)	DFR2	3 (0.5%)
D positive (no variant)	86 (15.5%)	DAU4	1 (0.2%)
D negative (no variant)	40 (7.2%)	DNB	3 (0.5%)
D negative rG	16 (2.9%)		

As reported in Table 1: 86 subjects (15.5%) were D positive and 56 (10.1%) were D negative, without variant, in 16 subjects (2.9%) a rhG genotype was observed.

In 362 subjects (65.2%) we observed a D weak and in 43 subjects (7.7%) a D variant was detected.

Considering D weak subjects, as reported in Table 1, the more common D weak type observed was the D weak type 1 (227 observations, 40.8%) followed by D weak type 11 (47, 8.4%), type 2 (46, 8.3%) and Type 15 (34, 6.1%). Of note is the observation of 4 (0.7%) subjects carrying the D weak type 18 which is quite uncommon in the Caucasian population.

Considering the D variants, the most frequently observed genotype, as expected, was D partial type VI (27, 4.9%), followed by D partial type V (4 cases, 0.7%) and D partial type III and D Partial type DFR2 (3 observations, 0.5% each).

In our experience none of the subjects with weak D developed allo-antibody with anti-D specificity, however among the subjects with variant D we observed one subject, a pregnant woman with a D variant type DAR, who developed an allo-antibody with anti-D specificity.

4. DISCUSSION

This study was conducted in North-East Italy in a population predominantly (94.%) composed of individuals of Caucasian ethnicity. This fact must be taken into consideration because prevalence of serological weak D phenotypes differs with race/ethnicity: in the Caucasian population, the prevalence of serological weak D phenotypes is estimated to be relatively high, ranging from 0.2% to 1.0% while , it is tenfold lower in the Asian population. In addition, different RhD serotyping methods could result in substantially different estimates rates for the prevalence of serological weak D phenotypes [18,19].

Interpretation of D type in blood donors can be complex, because some variants of the D antigen with low antigenic density can be undetectable by methodologies with relatively low sensitivity. Consequently, blood components can be mislabelled as RhD-negative, exposing RhD-negative patients to the risk of anti-D alloimmunisation. In serological investigations, the main difficulties occur in oupatients with sickle cell disease who are of African descent and with great miscegenation, which makes the presence of non-detectable RhD and RhCE variants common. The same occurs in patients with oncological diseases and other haematological diseases who receive periodic transfusions because of chemotherapy. In general, the *RH* variant is suspected only after alloimmunisation. In this study, among the considered population, we found weak D reactivity in 555 samples, in one or both clones tested, and reactivity discrepancy between the two clones in the same sample. Routine serological techniques are not able to differentiate between weak D and partial D, but they detect the weak expression of the D antigen, suggesting the presence of *RHD* and *RHCE* variant alleles. Subjects, blood donors and/or patients, with this condition should be studied molecularly [20,21].

The first consideration that emerges when examining the results obtained in our study is that the selection criterion adopted to identify the samples to be subjected to RHD genotyping on the basis of the phenotyping for the D antigen does not appear to be completely adequate. In fact, out of 555 samples sent for genotyping, 86 (15.5%) were D positive and 40 (7.2%) were D negative, without RHD genotyping allowing the highlighting of D weak or D variant. As regards the 86 D positive subjects, who were genotyped,

without evidence of D weak or D variant, this observation is not surprising as the literature has well reported the possibility of observing a weakly expressed D in serology, as a consequence of a gene interaction between the RHC and RHCE alleles [22,23]. As regards the 40 D negative subjects, who were genotyped, without evidence of D weak or D variant, this is a consequence of our sample selection policy. Usually, the majority of D+ red cells show clear macroscopic agglutination after centrifugation and can be readily classified as D+. Red cells that are not immediately or directly agglutinated cannot as easily be classified. For some D+ red cells, demonstration of the D antigen requires incubation with the anti-D reagent and addition of antihuman globulin (AHG) serum after incubation with anti-D [24]. These cells are considered D+ (weak), even if an additional step in testing is required. In our Laboratory in presence of D weak observed only after execution of an assay with Anti Human Globulin (AHG), a Direct Antiglobulin Test (DAT) is performed to evaluate presence of interfering auto antibodies. If DAT I positive results is considered uninterpretable and the sample is selected for RHD genotyping. This policy of selecting samples to be subjected to RHD genotyping can, in our opinion, explain the relatively high percentage of D negative subjects subjected to diagnostic analysis in molecular biology [25].

Studies conducted in Europe analysed the frequency of *RHD* alleles and found that 95% of Caucasian individuals with weak D antigen expression are RHD weak D type 1 to 3 [26-28]. In other studies, in the Brazilian population, *RHD*weak D type 1* was the most frequently found variant [29]. Results obtained in this study are partially in agreement with the data reported in the literature, in fact of the 555 subjects examined, 362 (66%) showed a weak D after RHD genotyping. In the considered series, the most frequently observed D weak was D weak type 1 (227 cases, 40.8%), as expected in the Caucasian population. D weak type 11 (47 cases, 8.4%) and D weak type 2 (46 cases, 8.3%) followed in frequency, by D weak type 15 (36 cases, 6.1%). However, no case of D weak type 3 was observed. Of note were 4 cases (0.7%) of D weak type 18 which is considered rather rare in the reference population. As expected, none of the subjects with weak D developed antibodies with anti-D specificity. As matter of facts in 2015, the American Association of Blood Banks (AABB) College of American Pathologists (CAP) Working Group recommended that *RHD* genotyping be performed in patients with a serological weak D phenotype, as patients carrying any of the three most prevalent alleles in Caucasians with a serological weak D phenotype (RHD*01W.1, RHD*01W.2, and RHD*01W.3) can be safely managed as D-positive. This practice allows optimal allocation of scarce D-negative RBCs and prevents unnecessary administration of Rh immune globulin (RhIG). More recently, members of the AABB-CAP Working Group have updated their recommendations that patients with the RHD*09.03.01 (weak D type 4.0) or RHD*09.04 (weak D type 4.1) allele also be managed as D-positive [30-33].

In our series only 47 subjects (7.8%) had a D variant after RHD genotyping. As expected, DVI was the most frequently observed D variant (27 cases, 4.9%). DVI is considered a clinically significant variant since DVI+ subjects can also produce antibodies with anti-D specificity following immunotherapy, transfusion or during pregnancy. In our laboratory we actively search for the DVI variant with serological methods using gel card tests with two anti-D microtube, one of which is capable of specifically recognizing the DVI variant [33]. This screening method has proven to be extremely sensitive (1.00) and specific (1.00). In fact, genotyping for RHD confirmed the presence of a DVI variant in all 27 samples identified by the serological method. While in none of the samples not identified by serology screening , we identify a DVI variant by RHD genotyping. In our series of patients we found the formation of a specific anti-D allo antibody in a woman in her second pregnancy carrying a D variant type DAR [34,35].

This study has some limitations: it is in fact a single-centre retrospective study; the selection of samples to be subjected to RHD genotyping was carried out based on the results of serological tests. Both donors and patients were selected. It was therefore not possible to establish a prevalence data of the forms of weak D / variant D in the reference population but only an analysis of the frequencies of the different forms of D variants / weak D observed.

5. CONCLUSIONS

Approximately 0.2% to 1% of routine RhD blood typings result in a serological weak D phenotype. In the era of “phenotyping only” assays, serological weak D phenotypes have been managed by policies to protect RhD-negative

women of child-bearing potential, as well as patients who will be candidates for chronic transfusion therapy (hemoglobinopathies, leukemias, etc.) from exposure to weak D antigens. Typically, blood donors with a serological weak D phenotype have been managed as RhD-positive, in contrast to transfusion recipients and pregnant women, who have been managed as RhD-negative. RHD genotyping allow a simple identification of D weak / D variant. Most serological weak D phenotypes in Caucasians express molecularly defined weak D types 1, 2 or 3 and can be managed safely as RhD-positive, eliminating unnecessary administrations of Rh immune globulin and conserving limited supplies of RhD-negative RBCs.

CONSENT

Each subject gave written consent to be subjected to serotyping and for any further investigations by genotyping.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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