Review Article

Serological analysis of Rh antigens: how far can we go?

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Abstract: The Rh blood group remains the most clinically significant blood group system, next to the ABO system and it is one of the most complex among the systems. Proper matching of Rh antigens between donor and recipient is essential to prevent risk of alloimmunisation and haemolytic transfusion reactions (HTR), especially among vulnerable groups. Antibodies to Rh antigens are also one of the leading causes for haemolytic disease of foetus and newborn (HDFN). Serological typing of Rh antigens plays an indispensable role in blood transfusion practices. Accurate determination of the Rh status of blood donors, pregnant mothers and potential recipients of red cell transfusions is crucial for ensuring safe transfusion and prevention of HDFN. The ready availability of monoclonal antibodies with high sensitivity and specificity has facilitated the accurate identification of the principle clinically significant antigens of the Rh blood group system—D, C, c, E and e. However, the Rh blood group system is characterised by numerous RHD and RHCE variants that result from nucleotide variations and gene rearrangement events involving the corresponding *RHD* and *RHCE* genes. Identification of these variants is important in the clinical setting and the serological approach to their recognition may differ in different context such as between being a blood donor and a patient. Knowledge of the monoclonal antibody clones used for typing and their reaction patterns provides useful information to deduce the underlying variant. Molecular typing is however often required to resolve serological discrepancies or to confirm the serological deduction.

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Introduction

The antigens that make-up the Rh blood group system, in particular the D, C, c, E and e antigens are highly immunogenic and can be the source of immunisation and subsequent haemolytic transfusion reactions (HTR), when antigen positive red cells are transfused to a corresponding antigen negative subject. It is also a common contributor to haemolytic disease of foetus and newborn (HDFN).

In 1940, Landsteiner and Wiener, initially observed that antiserum, obtained from guinea pigs and rabbits immunised with Rhesus macaque red cells, agglutinated about 85% of the human red cells they tested, and their reactivity pattern closely paralleled the reactivity seen with serum obtained from women who had pregnancies complicated by haemolytic disease of newborn (1). The proposed antigen on human red cells that the anti-serum from Rhesus macaques reacted to, was duly referred to as the Rhesus antigen and the antibody as 'anti-rhesus'.

It was somewhat during the same period that Levine and Stetson described a case of erythroblastosis fetalis that was ascribed to the red cell isoimmunisation of a mother by a paternal red cell antigen, which was subsequently identified as the 'Rhesus' antigen (2). Later work however confirmed that the 'anti-rhesus' that Landsteiner and Wiener had identified was anti-LW, and the confusion arose because the LW antigen is more strongly expressed on Rh-positive cells then Rh-negative red cells (3).

The controversies and confusions within the Rh blood group system continued for the next few decades following recognition that 'Rh-positive and 'Rh-negative' was due to the presence and absence of the D antigen, and that there were additional antithetical antigens, designated as C, c and

E, e linked to the D antigens (4). The divergent opinions among groups with regards to the expression and the number of genes encoding these closely linked antigens, was reflected in the different terminologies and nomenclature proposed to notate the antigen combinations (5,6).

While the Fisher-Race terminology was based on the proposal that three closely linked genes C/c, E/e and D conferred the different Rh phenotypes, the Wiener nomenclature took the opposing view that a single gene encoded the different rhesus blood group factors. Both the theories were however found to be incorrect, and it was the proposal by Tippett that the various Rh antigens can be explained by the interaction of just two genes, *RHD* and *RHCE* that was subsequently proven to be correct (7). It took several decades of concerted efforts by different groups to unravel the serological, biochemical and molecular basis of the Rh blood group system, which remains the most clinically significant blood group system next to the ABO.

The nomenclature used in this review article follows the general guidelines for naming of blood group alleles (https://www.isbtweb.org/resource/isbt-guidelines-naming-blood-group-alleles.html) and for the naming of RH alleles (https://www.isbtweb.org/resource/guidelines-naming-rh-alleles--pdf.html) as outlined by the International Society of Blood Transfusion (ISBT).

Molecular basis of Rh antigens

The antigens of the Rh (ISBT 004) blood group system are encoded by two genes, *RHD* and *RHCE*, located at the chromosome locus 1p36.11. Both genes encode 417 amino acids and show close homology. They occur in inverted orientations, contain 10 exons each and are 97% identical (8-10).

RHD expresses the RhD polypeptide responsible for conferring D antigenicity. The *RHCE* meanwhile encodes the RhCE polypeptide, responsible for the common antithetical C/c, and E/e antigens. These antigens result from several single nucleotide variations that cause amino acid substitutions and epitope conformational changed of the RhCE protein. Specific combinations of these amino acid substitutions within the same polypeptide can additionally express compound antigens such as Ce, CE and ce (referred to as f). The Rh blood group system is complex, and various antigens are generated by combinations of single nucleotide variations and gene rearrangement events (11). To date, there are 60 ISBT recognised antigens within the Rh blood group system (https://www.isbtweb.org/resource/tableofbloodgroupsystems.html).

D-negative

D-negative individuals do not express the RhD polypeptide, either due to deletion of the *RHD* gene or transcriptional silencing of the gene. *RHD* deletion is the common basis for the D-negative phenotype among those of Western descent (12), while deletion and silencing mutations contribute to the D-negative phenotype in Asians (13). Among those of African descent, inactivation of *RHD* commonly results from a 37-bp internal duplication in exon-4 leading to a premature stop codon at position 210 (14). The D-negative phenotype in all populations occurs due to the absence of D antigen, and consequently, there is no antithetical 'd' antigen. D-negative individuals can produce anti-D, which is essentially a polyclonal mixture of antibodies directed against various epitopes of the D antigen, when they are exposed to the complete D protein expressed on D-positive red cells.

In contrast, the C/c and E/e are antithetical antigens and therefore antibodies to the antithetical antigen are produced when an individual is exposed to red cells with a different C/c or E/e makeup to their own.

The strength of expression of the D antigen on the red cell surface is dependent on many factors that include transcriptional efficiency of the alleles, translational and trafficking of the protein to the surface and stability of its surface expression. These factors may be affected by nucleotide variations causing amino acid substitutions within the coding regions, principally effecting sequences that are embedded intracellularly or in the transmembrane regions, therefore altering the insertion of the RhD polypeptide into the red cell membrane (15,16). Such mutations cause weak D expression and are classified into the various weak D types, based on their degree of weakened expression and underlying molecular basis.

The D antigen may also be expressed at extremely low levels that is undetectable by routine D typing methods, including the D-weak test (17,18). Such red cells are designated as the Del. This is more commonly seen in apparent D-negative individuals in the East Asian population, where the *RHD 1227A* mutation effecting RHD mRNA processing is often identified (19).

D and CE variants

The *RHD* and *RHCE* genes show considerable sequence

similarity as they are of a common ancestral origin, likely having undergone gene duplication and diversification (20). The high homology and the adjacent locations of the two genes in the opposite orientation facilitates gene conversions and crossovers to produce hybrid genes that express portions of the RhD and RhCE polypeptide, with loss or gain of epitopes that are recognised by antibodies (15). These D-variants are often called partial-D although it is to be recognised that there is considerable overlap between D-variants causing a weak-D and partial D phenotype. Similar alteration may also give rise to the partial and weak phenotypes of the antigens normally expressed by the RHCE polypeptide (21,22).

Rh typing reagents

Despite widespread molecular characterisation of Rh antigens and their variants, serological typing remains an important cornerstone and is the primary investigational approach in the transfusion laboratory. The availability of good quality anti-sera with high sensitivity and specificity at reasonable cost coupled with reproducible laboratory techniques that are easily learned, makes serological typing an indispensable tool for characterisation and solving clinical and laboratory problems associated with Rh related antigens.

Polyclonal antisera

Following the discovery of the 'Rh blood type', considerable efforts were made to derive effective typing reagents, usually from a sensitised human individual or immunised animal. These reagents were obviously of polyclonal origin, principally of IgG subtype with wide lot to lot inconsistencies. Ensuring adequate avidity and specificity with stable continual supply was an issue (23-25). The antisera furthermore needed to be suspended in high-protein solutions to ensure avidity and extend storage (26). Despite the limitations, it is well recognised that most of the discoveries related to the Rh blood group system during a major part of the twentieth century, and the dramatic prevention of consequences from Rh related HDFN, were driven by polyclonal typing reagents and laboratory ingenuity. The importance of polyclonal antisera obtained by immunisation remains until now due to its broader range of reactivity, although it is not the principal type of antisera used in routine laboratory practise.

Monoclonals antibodies

Monoclonal antibodies produced from Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines revolutionised blood group testing and has had major implication in Rh typing (27-33). The monoclonal antibodies, unlike their polyclonal counterparts, have high specificity and sensitivity, strong reaction patterns, shorter incubation time, and are more consistent, giving reproducible results from lot to lot (34). The availability of IgM monoclonal antibodies also allows for direct typing instead of relying on an indirect anti-globulin test when using polyclonal predominantly IgG antibodies (35-38).

Recognising the inherent limitations in human B-cell immortalisation and low EBV transformational efficiencies, more advanced techniques such as phage display have been developed to produced monoclonal antibodies to specific epitopes of Rh antigens that previously could not be achieved (39).

Monoclonal specificities

A variety of monoclonal antibodies, produced commercially or in-house are currently used for routine typing of the D, C^w, C/c and E/e antigens as well as some rarer antigens (34,40). Most commercial anti-D reagents currently are composed of a blend of two monoclonal antibodies from two different clones, or a monoclonal/polyclonal blend.

Monoclonal antibodies by virtue of them being derived from a single lymphocyte clone, are epitope specific, and therefore highly specific. It may however be non-reactive or show reduced avidity for Rh variants that have loss or conformational change of epitopes which act as the recognition site of the antibody. Different clones of anti-D may recognise different epitopes of the D protein, and consequently give rise to different reactivity patterns in D-variant red cells where certain epitopes may be altered or missing (41). It is also important for users to be aware that different manufacturers may utilise similar clones in their formulation and therefore a different make of anti-D monoclonal reagent may not necessarily mean different epitope specificity (40).

Rh proteins are conformation-dependent and are influenced by other proteins and lipids that are present on the red cell membrane. When defining D epitopes using monoclonal antibodies, it is therefore important that the

testing is performed at the correct pH, temperature, ionic strength, and antibody concentration as well as to always use red cells that have been stored correctly (42).

Applications of serological typing to the Rh antigens

Typing for the D antigen among blood donors, patients and pregnant women is mandatory in most countries to prevent the complications associated with Rh, particularly D-immunisation which includes HTR and HDFN. Countries with a homogenous population that exhibit low prevalence of D-negative subjects have proposed that D-typing may be omitted (43). However, with widespread migration and inter-mixing of population, it may be difficult to advocate such a policy these days.

Rh typing of blood donors

Among blood donors, it is important to identify all individuals expressing D as D-positive, regardless of the antigen density and even if the D antigen is incomplete (44). Highly potent monoclonal anti-D blends can achieve this objective since they are able to give a positive result even with weak D-variants (45,46).

It is still recommended though, that apparent D-negative donors should be subjected to a weak D test. Molecular typing strategies have been proposed to replace this standard serological approach to D negative donors (47), but this is not commonly practised. In the weak D test, the apparent D-negative red cells are incubated with a suitable IgG anti-D reagent, followed by an indirect antiglobulin test (IAT). A positive IAT would indicate that the red cells do express D antigens that have bound the IgG anti-D reagent, and therefore despite testing as D-negative cells on direct typing, the cells are designated as weak D and the donor should be designated as D-positive (44). Conventional serological techniques using readily available reagent antibodies are in most parts sufficient to identify weak D phenotypes (48). Blood donors identified as weak D may be subjected to molecular analysis, although this may not be required in the blood donor setting (49).

Among East Asians, the Del phenotype may account for many apparent D-negative reactions on direct typing. Del red cells are negative even on weak D testing but may be identified as expressing D antigens by an adsorption-elution procedure. It is reported that 23.3% of Chinese of Han ethnicity and 10% of Thai individuals who type as RhD negative are of Del phenotype (18,50).

The majority of Asian Del is due do the *RHD 1227A* mutation (18,50,51), which tends to be associated with the r' (Ce) phenotype, and therefore subjecting apparent D-negative donors who are C-positive, to an adsorption elution test or molecular typing has been recommended (52-54). This is to avert the risk of designating a Del donor as D-negative and potentially causing D-immunisation in a D-negative recipient of the blood (49,55,56).

The identification of partial D is particularly important in the blood donor setting, so that the donated blood units can be designated as D-positive and not made available to D-negative recipients (57). Careful consideration of the reagent antibodies used to type blood donors is therefore important to ensure that their specificities can detect partial D types that may be prevalent or of clinical significance.

It is well recognised that despite the provision of Rh antigen matched red cells, some recipients may continue to develop Rh related alloantibodies. This can be attributed to the recipient harbouring a partial Rh antigen, or to the transfusion of red cells containing a variant Rh antigen that is not recognised during donor typing and subsequently inducing immunisation in the recipient (57). Patients with sickle cell disease receiving repeated transfusions are especially at risk of this complication (58,59).

The recommended approach for blood donor typing is that they are tested using two different D typing reagents of different clone compositions, so that a discrepancy or weakened reaction pattern can alert to the possibility of an underlying D variant or rare Rh type. Combination of two anti-D typing reagents, each usually composed of two of more clones, is therefore preferred, as individual monoclonal anti-D reagents will not be able to detect all partial D donors reliably (42,60,61). Such strategies are effective in identifying potential D variants among blood donors who can then be further typed through molecular methods for confirmation (62,63).

Among Caucasians, the DVI category is considered the most clinically relevant, and therefore one of the anti-D used should positively identify DVI (64). This ensures that donors who are DVI are not inadvertently labelled as D-negative due to non-reaction from a clone that is directed against the missing epitopes missing on DVI red cells. In other populations where DVI may not be the most clinically significant D variant, consideration should be given to develop other combinations of monoclonals.

Serological typing also has an important role in screening blood donors to identify donors with potentially

immunising Rh variants, who can then be subjected to molecular typing for characterisation of the variant. The diversity of Rh variants among those of African origin, may especially pose a problem, when their donated red cell units are transfused to recipients lacking the Rh variant antigens (65). On the other hand, identifying donors lacking certain antigens such as hr^B (RH31) and hr^S (RH19) would be beneficial to support similar antigen negative patients. Discrepancies in D typing may not only reflect D variants but may also be observed with RhCE variants, with one study describing that 94.9% of their partial D samples revealed *RHCE* variant alleles (66). Combining information from self-declared ethnicity and Rh phenotyping improves the yield for identifying Rh variants by molecular typing among blood donors in a heterogenous population (67).

Rh typing of pregnant women and potential red cell recipients

The primary purpose of D typing in potential recipients of red cell transfusions, is to ensure that individuals who do not express the D antigen or have portions of the D antigen altered or missing receive D-negative red cell units to prevent D immunisation (68). Pregnant women who are D negative or partial D, should be eligible for routine antenatal anti-D prophylaxis (RAADP). Individuals who are weak-D expressing the complete D protein on the red cell surface, are generally considered safe to transfuse D positive red cells (69-71). Certainly, persons with weak D type 1, 2 or 3, all of which identifiable on typing with current monoclonal reagents, do not make anti-D on exposure to D-positive red cells. Other weak D types, such as weak D Type 4, however has been associated with development of anti-D in the patient (72).

Patients and pregnant women with the 'Asian type' *RHD1227A* Del phenotype do not make anti-D and therefore can be transfused with D-positive blood and antenatal anti-D prophylaxis withheld (73). However, patients with Del phenotypes due to other mutations have been reported to make anti-D and should therefore be considered as D-negative, with regards to their red cell transfusion management (74).

The general recommendation has been that a single anti-D reagent is sufficient for D-typing of patients and that serological weak D phenotypes are interpreted as D-negative for pregnant women and transfusion recipients. Among populations with relatively higher prevalence of DVI, the chosen anti-D should not react with DVI red cells. This avoids partial D individuals from inadvertently being classified as D-positive (42,75). However, other partial D phenotypes seen such as DIV, is also of clinical relevance and may need consideration when developing D typing guidelines for pregnant women and red cell recipients (76).

Patients with sickle cell disease of African origin, would benefit from molecular typing once there is an atypical or discrepant result on routine D typing, in view of them potentially being Rh variants, examples of which include DIIIa, DAR and DAU (77,78). These variants may present with atypical reaction patterns on serological typing depending on the antibody clone used, but relying on serological typing alone for identification of this Rh variant is not possible (79). Rare Rh types may sometimes show discrepancies in reaction patterns between monoclonal reagents. For example, the rare RH33 (R_0^{Har}) antigen associated with the DHAR type, is reactive with IgM clones GAMA-401, TH28 and MS201, while negative with the MAD2 clone (40).

Failure to identify an expectant mother as a D variant and instead labelling then as D-positive can lead to risk of Rh immunisation from transfusion of D-positive red cells, or failure to prevent HDFN due to omission of antenatal anti-D prophylaxis. Serological typing for Rh antigens is therefore important and identification of any discrepancies or weakened reaction should be an alert for further investigations (80). HDFN has been associated with anti-D developing in mothers with D variants such as DVI, DBT, DIVb, partial Del (81) as well as a large range of antibodies to rare Rh antigens (82).

Most guidelines would recommend that samples from pregnant women and potential recipients of red cell transfusions, that give a result of less than 2+ on serological testing with monoclonal anti-D be investigated further for D variants. Molecular typing is usually recommended for identification of individuals with weak D type 1, 2 or 3, who do not develop anti-D and prophylactic anti-D can be conserved (83). Serological typing using a selection of monoclonal antibodies may however aid in the distinction as proposed by some authors (84,85).

In the era of molecular typing, policies with regards to how to manage patients and pregnant women with discrepant Rh typing results, should be decided at a local level and coordinated at the national or regional level, taking into consideration the population makeup, diversity of various Rh phenotypes and their variants, and the risk of alloimmunisation to Rh antigens as a consequence (48,72). Migration patterns will also need to be taken into account when developing such policies (86).

Resolving Rh typing discrepancies

Discrepancies in D typing may be observed as either an atypical weaker than expected reaction or a difference in the reaction pattern between two different reagents (87,88). Weak D phenotypes are often characterised by weaker than expected reaction patterns and may show distinct phenotypic pattern with different reagent antibodies (89,90). Discrepant or atypical weak results between D typing reagents using different monoclonal antibodies may indicate partial D or a weak partial D variant and should be further investigated by serological and molecular approaches (91). Serological analysis should include further phenotypic characterisation using a panel of antibodies to the D, C/c and E/e antigens as well as antibodies that recognise different epitopes (epD) of the D protein, if available (90,92).

Commercial kits with a limited number of 6 to 12 monoclonals to epD are available in the market for basic characterisation of the partial D type. However, the wide availability of molecular typing and next-generation sequencing would likely make this approach a more feasible option for defining the underlying mutation and confirming the allele responsible for the phenotype.

In an Egyptian study, serological analysis using six monoclonal anti-D was performed on 50 samples that showed poor reactivity with anti-D (93). Serological analysis alone was able to identify 17 of the samples as partial D, while molecular analysis resolved 24 of the remaining 33 cases, of which 23 of the 24 were weak D types. Among 23 blood donors from a blood centre in Morocco, who typed as weak D, complete epD profiles was observed in 16 of them, of which 11 were identified as weak D type 4 by molecular typing and one each of weak D type 61 and DAU0 (94). Three samples of complete epD with weak D expression remained unresolved. Of the 7 with incomplete epD profiles, there was 1 each of weak D type 1, 2 and 3, and a DOL1 and DVII. Kulkarni *et al.* reports the characterisation of 60 anti-D-discrepant samples using a kit with 12 monoclonal anti-D reagents. Of the 60 samples, only 7% could not be characterised (85).

Monoclonal reagent reactivity patterns may also facilitate the identification of novel Rh antigens. The Rh antigen, Crawford, was identified by recognition of a reaction with certain anti-D clones such as GAMA-401, but not with the other clones (95,96). Several RHD and RHCE variants can be identified by variability in reactions with various monoclonals (41,97). Recognition of such variants are of particular importance among Africans, especially in patients with sickle cell disease, requiring repeated blood transfusions (98).

Discrepancies in reaction pattern may occur not only between different monoclonal antibodies, but also between techniques. Examples of different reactivity patterns in weak D types 1 and 2 have been reported between different clones of anti-D as well as between serological testing using tubes and column agglutination technology (99). Tube tests generally have a higher degree of subjectivity due to the many technical variables in its performance as compared to column agglutination technology.

These studies illustrate the utility of serological techniques in resolving discrepant results, especially when employing a comprehensive monoclonal antibody panel. Nevertheless, molecular typing would continue to have a complementary role in serologically unresolved cases or to confirm the serological findings.

Issues and pitfalls in serotyping

Serological typing of Rh antigens has stood the test of time, within the scopes of routine blood donor typing as well as patient and antenatal testing. It is an efficient process, open to automation, sensitive and cost-effective. Current monoclonal typing reagents for D, C, c, E and e show sensitivities and specificities approaching 100%. False positive reactions are rare with Rh directed monoclonal antibodies although anecdotal cases have been reported (100).

Precise identification of Rh variants however remains a limitation using serological typing alone. The availability of specific monoclonals to a range of epitopes expressed by the Rh proteins has contributed to effective mapping and classification of the various variants, although this is not widely done outside of reference laboratories. In routine practice though, molecular characterisation has become a more widespread approach (79,92).

Nonetheless, it is still important to note that comprehensive serological typing should precede or accompany any molecular typing. False positive results for negative phenotypes may be generated by molecular methods when the testing strategy does not include identification of mutations in regions that involve transcriptional control of the *RHD* and *RHCE* gene, and accessory genes such as *RHAG* that is required for expression of the Rh proteins. False negative results with molecular typing may also occur when primers designed for identification of specific alleles do not take into account

potential rare nucleotide variations or hybrid alleles (101).

Serological typing methods however are limited in certain clinical circumstances, such as when there are mixed population of red cells as in post-transfusion (102), or when there is a need to determine zygosity. Rh monoclonal antibodies are generally effective as typing reagents even in antibody sensitised red cell which are direct antiglobulin test positive, such as in autoimmune haemolytic anaemia and HDFN (38). Occasionally though, heavily sensitised red cells may block the reactivity of typing reagents giving rise to a false negative serological typing result (103).

Molecular typing of *RHD* and *RHCE* is also recommended for patients with sickle cell disease who are regularly transfused, to detect rare RhD and RhCE antigens or variants, which can pose an alloimmunization risk if the patient is transfused non-genotype-matched red cells. These rare antigens and variants are often missed on routine Rh typing (104).

Conclusions

Serological typing of Rh antigens in routine donor and clinical settings is well entrenched and will remain so for many decades to come. The technique is reliable and cost-effective, simple, inexpensive and easily interpreted. It is easily accessible even in regions with limited resources. However, the huge diversity of Rh antigens, and the myriad rearrangements between the *RHD* and *RHCE* genes producing the Rh variants, may pose a problem with interpretation of serological typing results. Unusual Rh antigens and variants oftentimes can yield misleading and inconclusive results.

Over the past couple of decades following the wide availability of molecular tools, debates have been generated on the merits and demerits between serological and molecular typing. Molecular typing has emerged as a powerful tool, even in complex and challenging clinical scenarios, providing high accuracy and specificity in identifying the molecular basis for Rh antigens. When approached correctly, it can detect rare and variant antigens, leading to the resolution of many perplexing serological problems as well as improve blood donor management, transfusion practice and prenatal testing.

Serological typing will certainly remain the mainstay of transfusion testing, but it is the combination of both serological and molecular approaches that will offer the most comprehensive solution to meet the needs of the transfusion laboratory as well as the blood donor and patient.

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