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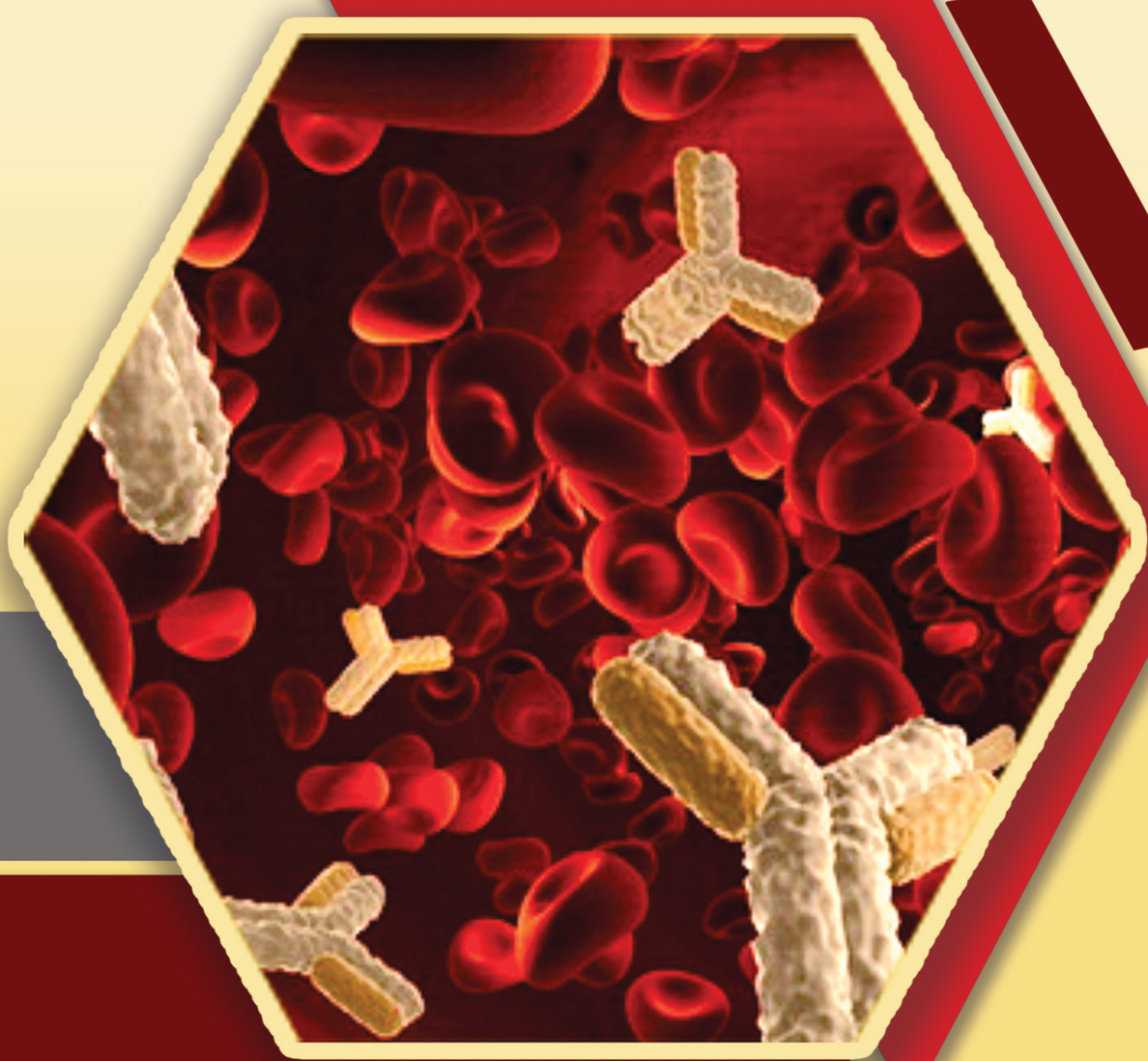

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Serological & molecular techniques for RhD typing and their role in the identification of D variants

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Protein based Rh blood group system contains over 50 antigens, the most important of which are D, C, c, E and e. These antigens are known to maintain the integrity of the RBC membrane and their absence from the RBC surface can cause mild haemolytic anaemia, membrane abnormality and change in cationic permeability. The immunogenicity of D antigen is 20 times more than c, the second most potent Rh antigen. C and E antigens are antithetical to c and e antigens respectively.

Based on the D antigen expression on RBCs, individuals are categorised as RhD positive and negative. The frequency of D positive individuals varies in different ethnic groups i.e. 85% in Caucasian, 92% in African, 99% in East Asians and 93-95% in Indians (1). In D negative individuals, the RhD protein is not expressed due to homozygous deletion of the entire RHD gene, formation of RHD-RHCE hybrids, involvement of various InDel or SNP mutations. To determine the expression of D antigen on the RBC surface, the cells are serologically typed with anti-D reagents. These reagents can screen one or more epitopes of RhD protein. In 1946, Stratton assigned the term Du to the red cells that were agglutinated by some anti–D but not by others. This definition was based on the results of tests using anti–D from individual donors. With the advent of polyclonal reagents made from pool of antisera containing anti–D, Du refers to cells that are not directly agglutinated with anti–D reagents as they carry less quantity of D antigen. The D antigen in these variants are detected by sensitive technique like Indirect antiglobulin technique (IAT).

Thus, Du phenotype represents weakened expression of D antigen. Different grades of Du have been reported by routine serology, the lowest being detectable only by IAT and highest reacting with some, but not all anti–D sera. Very low grade Du can not be detected by routine serology and requires sensitive technique like adsorption - elution. In the early 1950, several reports described D positive individuals who made anti-D, which reacted with all D positive samples

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except their own. Wiener and Unger (1959) postulated that the complete D (Rho) antigen has four parts designated as RhA, RhB, RhC, RhD that could be absent in rare instances and can produce alloantibody against the missing fraction, if exposed to red cells that possess the complete D antigen. Tippett and Sanger (1962) worked on reactivity of anti–D sera from Rh positive people and have led to a second method of categorizing the D mosaic. Nine epitopes of D antigen were recognized and based on reactivity seven categories of D variants were identified and designated by Roman numerals I to VII. The concept of epitopes emerged after the advent of monoclonal anti-D reagents, and now at least 37 epitopes of D antigen have been reported.

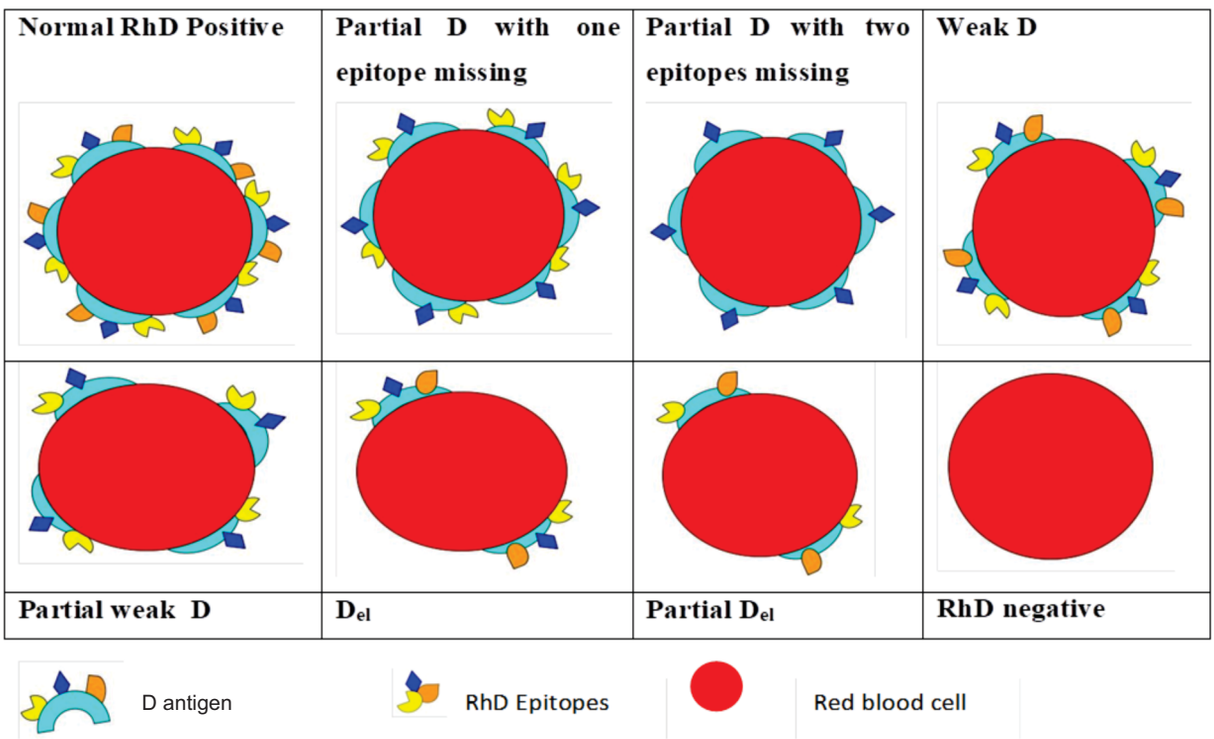

Figure1: Characterstics of Normal D & RhD variants

D variants can be quantitative or qualititative and are broadly categorised as weak D, Partial D and Del (Figure1). Weak D is a quantitative variant i.e. all epitopes of D antigen are present, but with reduced expression. RhD protein formed by partial D variants lacks one or more D epitopes. The expression of RhD protein encoded by D_{el} variants is so low that sensitive serology technique like adsorption elution is required for their identification. D variants are classified according to molecular classification i.e weak D 1, 2, 3 etc and partial Ds in different categories. Currently, 105 partial Ds and 155 weak D types are addressed in Rhesus database (http://www.rhesusbase.info/). The incidence of D variants have been reported as 0.2-1% in European population with weak D Type 1, weak D Type 2, DVI, DVII, DNB, DFR etc. as the common D variants. The major D variants found in the African

population are Weak D type 4.0, *DAR*, *DV* type 1.In China approx. 32% D negatives show Del phenotype with a common splice site mutation *RHD* 1227G > A (3). In India, more than 58% of D variants have weak D type 150 as the predominant *RHD* allele (4). Based on population specificity, a significant variation exists in the policies and procedures of D variant typing.

Clinical significance of D antigen

D antigen is very immunogenic and clinically significant due to its involvement in cases related to haemolytic disease of the foetus and newborn (HDFN) and haemolytic transfusion reaction (HTR). D negative woman can develop anti-D antibodies following sensitization during pregnancy, which can cause mild to severe haemolytic disease in subsequent pregnancies. Cases related to HDFN can be traced back to the 16th century. However, it was in 1939 when Levine and Stetson reported the role of Rh antigens causing HDFN (5). The formation of alloanti-D in a D negative woman post-delivery was observed. In 1953, Argall et al reported the formation of anti-D in a D positive pregnant woman later identified as *D* variant blood type (6). Subsequently, anti-D produced by many D variant pregnant women were described and majority lacked some epitope of *D* antigen. HDFN cases related to Rh sensitization in D negative women were reduced significantly after the implementation of anti-D prophylaxis i.e RhoGAM (RhoD immune globulin human) in the 1960s. Anti-D prophylaxis prevents Rh sensitization by binding to the fetal D antigens circulating in the mother's blood. As women with D variant phenotype can also produce alloanti-D, they should be considered as a recipient of RhoGAM following delivery of a D positive baby. These antenatal women require more doses of anti-D prophylaxis as compared to D negative women. For appropriate administration of Rho prophylaxis, it is essential to determine the correct RhD status of the antenatal women. In people of African descent where the incidence of D variants is high, the chance of being sensitized due to multiple transfusion in Sickel cell disease patients increases. Hence, in such patients, the correct identification of RhD status reduces the chances of alloimmunisation and helps in transfusion management.

To know the correct RhD status and to identify D variants, a series of tests must be performed. Various population-specific serological and molecular typing methods are adapted worldwide to determine correct RhD status.

A) Serological typing

Serological D typing is based on the principle of the *in vitro* hemagglutination reaction. In this reaction, D antigen if present on RBCs. binds to the antibody (anti-D reagent) forming agglutination. Based on the quantity of the D antigen, the agglutination reaction strength can be categorised into different grades (+4 to +vw) (Figure2).

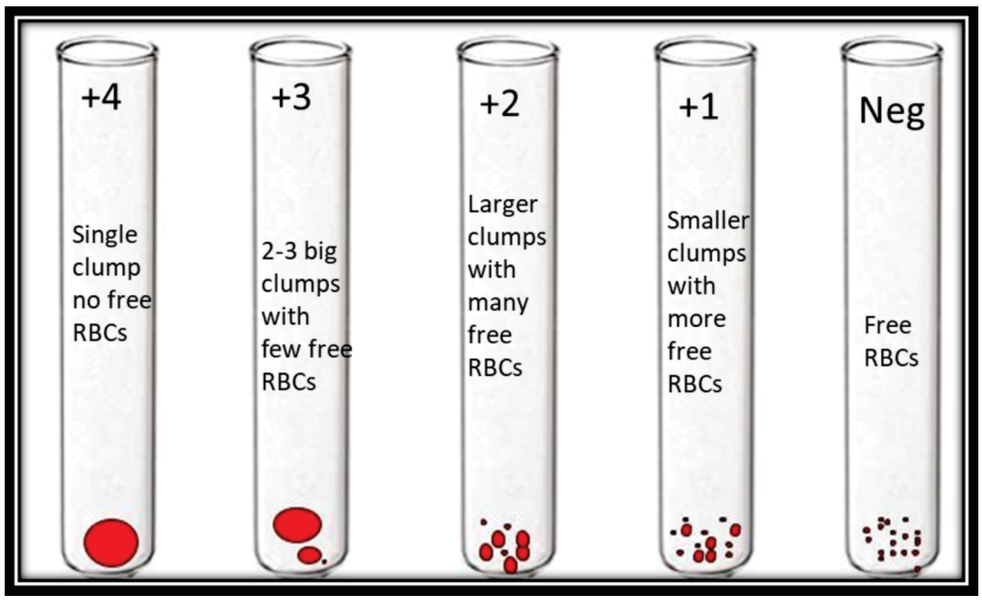

Figure 2: Agglutination grades recorded during serological typing of RhD protein

This agglutination reaction is easily visible if the reaction shows a +4 to +1 grade. However, if the number of D antigenic sites is far less giving +w to +vw reactions, microscopic examination is required.

Earlier only polyclonal anti-D reagents were available for D antigen typing. These reagents can be high (e.g. polyclonal IgG) or low protein-based, saline-based (e.g. IgM anti-D), chemically modified(7). The first available D typing reagent was saline-based which contained IgM anti-D and reacted in the saline phase. High-protein anti-D reagents derived from human plasma were introduced in the 1940s. These reagents could recognize multiple D epitopes due to their polyspecificity and thus could be used for weak D testing. To increase the sensitivity of anti-D reagents, Romans et al(1977) reduced interchain disulfide bonds of IgG antibody using dithioerythritol (8). This chemically modified IgG could freely form bridge between the red cells.

Initially, to screen the presence of D antigen, polyclonal anti-Ds derived from the plasma of alloimmunized individuals were used. With advent of hybridoma technology, the *in vitro* synthesis of monoclonal anti-D reagents using Epstein-Barr virus-transformed B-lymphoblastoid cell line came into existence. With monoclonal reagents, the sensitivity was increased and the incubation time of test was reduced. They are commercially available as IgM, IgG, or a blend of IgM plus IgG anti-D reagents. Based on the type of reagent used, there are two different approaches for D typing.

Immediate spin: Pentavalent IgM being a large molecule can easily detect D antigen in the saline phase. In RhD positive individuals, the number of D antigenic sites ranges from 10000-35000 per RBC. RhD blood group of the individuals expressing normal or high D antigenic sites can be easily determined using IgM anti-D reagents. In this method, an equal volume of 3-5% of RBC suspension is mixed with anti-D reagent and centrifuged (brings the antigen and antibody in close contact and assists in forming agglutinates).

Indirect antiglobulin test (IAT): D variants with less number of D antigenic sites (less than 10,000 per cell), may not be detected in the IgM phase and a more sensitive method like IAT is recommended. To perform IAT either IgM+IgG blend anti-D or IgG anti-D antisera can be used. If D antigen is present on the RBC, IgG anti-D forms antigen-antibody complex. A secondary antibody i.e. antihuman globulin (Coomb's reagent) is required to observe agglutination. The antihuman globulin reagent was earlier produced by immunizing mouse/rabbit with human globulins, but now available as monoclonal reagent. The serological techniques routinely used for RhD typing are as follows:

Conventional tube technique (CTT): For more than 80 years, CTT has been a gold standard technique for detecting blood group antigens. This method is still widely used due to its specificity and cost-effectiveness. In this method, an equal volume of the patient's RBCs suspension and antiserum (IgG, IgM or IgM+IgG) is added in a test tube. If *D* antigen is present on red cells, immediate spin at 1000 rpm forms agglutinates. By gently swirling the tube, the reaction grade can be recorded (Figure2). IAT must be performed for the samples showing a negative reaction. The patient's red cells are saturated with IgG anti-D. If blend anti-D is used, tube showing negative or weak reaction following direct spin can be further incubated at 37^{o} for one hour. Unbound antisera are removed by washing the cells with saline. Anti human globulin reagent is added, mixed and the tubes are centrifuged and the results are recorded. As the reaction grade determines the quantity of D antigen, it should be recorded correctly by skilled personnel. Weaker reactions may not be detected or may require prolonged incubation time for detection.

Column agglutination technique (CAT): To overcome the limitations of CTT, a new system based on sieving effect of glass - bead micro particles was developed. In this method, the gel acts as a sieve and allows free RBCs to pass through the pores while trapping only agglutinates. Today various commercially prepared gel cards are available and are popular due to simple & quick test, and documented interpretation of results. A gel card can be neutral or with antisera. Similar to the CTT, the strength of a reaction in CAT can be graded from +4 (band of red cells on top part gel column) to +vw(some agglutinates in lower part along with a clump of free RBCs). The reaction strength of gel cards can be visualized after several hours. Due to the high sensitivity, the gel cards can easily detect extremely weak reaction observed in D variants. Some gel cards coated with Anti-D IgG also allows

weak D testing. To perform weak D testing, 50 ul of 0.8 to 1% RBC suspension is added to the LISS/Coombs gel card coated with anti-IgG and anti-C3d.This is followed by addition of 25ul IgG anti-D reagent. After incubation at 37°C for 15 min, and centrifuging (10 minutes at 1000 rpm) the gel card, result can be visualized. Some commercially available cards specially identify common DVI variant in Caucasians. The red cell suspension added to the gel card is prepared in LISS (Low ionic strength solutions) to reduce the zeta potential and incubation time. Gel card testing can give a stronger reaction (≥3+ reactivity) with D variants which can be mistyped as RhD positive. Luo et al have recommended a two-method strategy i.e. gel card and tube testing for correct identification of D variants (9).

Microplate method

In 1966, a microplate-based system was introduced for the detection of blood group antigens to reduce the wastage of valuable cells or antisera. Similar to tube testing, a small volume of cells and antisera are added to the microwells. Later development like the use of polyethene glycol (PEG) medium was made to enhance the agglutination in liquid phase microplate system. Plapp et al. in 1986 developed a solid phase method that involved coating of antigen or antibody on a microplate (10). To check the presence of D antigen, 96 well microplates (modified polystyrene) is coated with IgG antibodies and red cell suspension is directly added to the wells. In the presence of D antigens, the cell adheres to the side of the wells and in the absence of D antigen, the red cells settle down and form a button after centrifugation. There are various automated systems like Capture-R™ system, introduced by Immucor Inc. (USA) and Solidscreen™ system by Biotest AG (Frankfurt, FRG) which have been adapted by many blood banks. However, due to the high sensitivity of the microcolumn, the cut-off score to differentiate *D* positive from *D* variants varies among laboratories. Some labs recommend a cut-off score of ≤ 2+ while others consider samples showing a score of ≤3+ on direct agglutination for D variant analysis (11).

Flow cytometry: Immunogenicity of D variants depends on the quality and quantity of D antigen on RBCs. For estimation of D antigenic sites, various methods like immunoradiometric assay (IRMA), Enzyme-linked immunosorbent assay (ELISA) and flow cytometry have been used. Historically, anti-D reagents were labelled with radioactive ^{125}I to determine D antigenic sites. To avoid the use of radioactive material, a fluorescent-based flow cytometric method was introduced which could count the fluorescence intensity of each red cell in a suspension rather than giving an average measurement over the whole population of RBCs. Flow cytometry works on the principle of hydrodynamic focusing and measures the fluorescence intensity of all the FITC (fluorescein isothiocyanate) labelled cells. In earlier tests, the *D* antigens were saturated with FITC labelled IgG fraction of human polyclonal anti-D, and fluorescence measured using Cytofluorograf SOH (Ortho Instruments). Later instead of polyclonal, monoclonal anti-D reagent and FITC conjugated antihuman IgG were utilized

for quantification of D antigen. Jones et al (1996) carried out quantitation of D positive and D variants samples(12). They found that number of D antigenic sites was highest in R_2R_2 (25,000) followed by, R_1R_1(15,000), R_2r (12,000) and R_1r (11,000) phenotype. Weak D samples showed 100 to 1300 D antigenic sites whereas depending on the D epitopes present in partial D variants, antigenic sites varied and in some were equal to D positive RBCs. The D antigenic sites decrease in the order of DVa > DIVa > DIII > DFR > DIVb > DVI in D variant individuals (13). Flow cytometry has gained popularity over the time due to its simplicity and cost-effectiveness to determine *D* antigen density, to resolve ambiguous D typing results, differentiate between weak and partial Ds and for detection of D+/D- chimeras.

Surface plasmon resonance

A surface plasmon resonance (SPR) array has been utilized to study the interaction of various biomolecules and drugs. For detection of RhD protein, IgG Anti-D (ligand) is immobilized on a carboxy modified gold layer sensor using a flow micro spotter. The cell suspension then flows over coated wells, which allows the generation of sedimentation response all over the region of interest (Figure3). An increase in the mass at the surface changes the refractive index, which changes the resonance angle (δθ) and the shift can be measured as a resonance unit(RU(Figure3)). The RU of normal *D* antigens, ranges from 530-1200RU and for negative cells show RU less than 100 (14). Weak D individuals show RU range 180-580 RU whereas in the case of partial D variants the range is 352 -1147 RU.

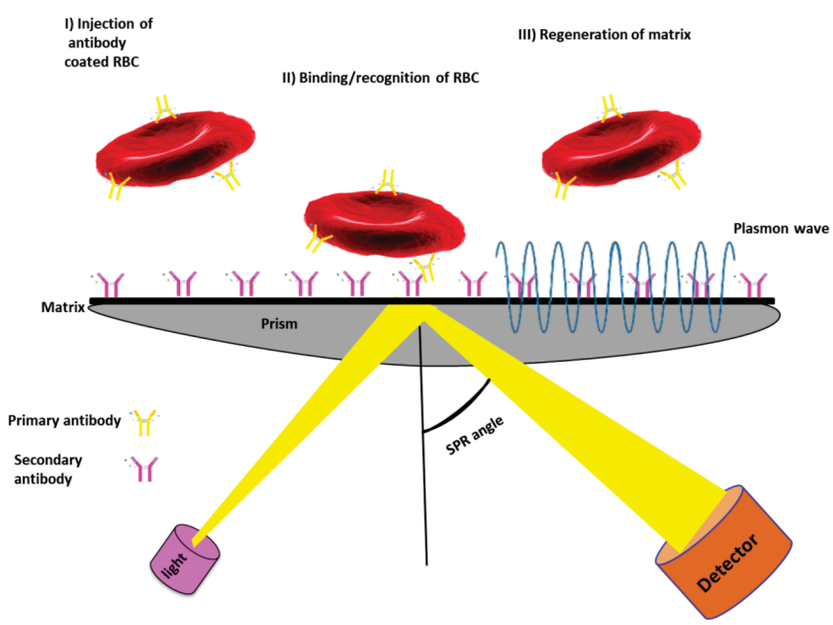

Figure 3: Diagrammatic representation of SPR role in determining presence of D antigen on RBC surface

A new approach to utilize SPR by simultaneous measurement of multiple blood groups including ABO and RhD within five minutes has been proposed (15). This method showed 100% concordance in the results of classical typing by serology and multiplex SPR imagining. SPR has an advantage over flow cytometry as it does not require binding of labelled ligands and the surface -sensor can be regenerated many times making it cost-effective.

Limitations of serological typing

Monoclonal anti-D reagents are produced against specific *D* epitopes and blended to prepare anti-D reagent. The specificity of these reagents varies based on the clones used to make antisera by the manufacturer. Partial D variants lacking some of the *D* epitopes can be typed as RhD positive/negative based on epitopes against which monoclonal anti-Ds are produced. These variants may show a strong reaction with some anti-Ds and a negative reaction with others depending on the type of antisera used by a laboratory. At least two commercial monoclonal anti-D reagents from different manufacturer or different batches of the same manufacturer is recommended for D typing. Some weak Ds and partial Ds can be serologically characterized depending on the reactivity profile with a panel of epitope-specific anti-Ds (commercially available).

Some D variants cannot be identified by IgM anti-D in immediate spin due to reduced expression of *D* antigen/epitopes. Therefore, in such cases use of IgG anti-D is recommended. Typing with IgG anti-D involves incubation of RBC with monoclonal IgG anti-D reagent followed by an IAT. Thus for samples showing negative reaction with immediate spin, a sensitive technique like IAT should be performed to detect reduced expression of D antigen in weak Ds.

D_{el} variants can escape identification by IAT as the expression of RhD protein is extremely low. These variants can be detected by a sensitive technique like adsorption elution. In this technique, D antigen is first saturated with IgG anti-D which then eluted by organic reagent like ether, which then thus indirectly predicating the presence of antigens. Adsorption elution test is not performed routinely.

Various commercially available serological kits allow the detection and characterization of several common D variants. ALBAclone Advanced Partial D Typing kit is a panel of 12 monoclonal anti-Ds which are commonly used to characterize Weak D type 1 & 2, DII & DNU, DIII, DIV, DV, DCS, DVI, DVII, DOL, DFR, DMH, DAR, DAR-E, DHK &DAU-4, DBT and RoHar in the western population. A report by Kulkarni et al (2013) showed the usefulness of this kit in the identification of D variants compared with the Diagnostics Scotland partial D kit (panel of 6 monoclonal anti-D)(16). The Indian D variants could be detected but not characterized using these typing kits.

Blood transfusion in a patient with D variant phenotype is challenging. Some D variants have been reported to produce anti-D after stimulus of *D* positive red cells and thus blood recipient individuals must be considered as D negative. Alloimmunization due to anti-D also varies based on the type of D

variants. Chances of forming alloanti-D against missing D epitopes are higher as compared to weak D or Del variants with all epitopes present. Though some weak D variants (Weak D type 1, 2, 4 etc.) are shown to form auto anti-D despite having all epitopes, a majority of the weak D variants have not been reported to produce anti-D and are managed as D positive. Thus in general, D variant individuals are considered D positive as donor and D negative as patient or recipient of blood transfusion. For D variant antenatal women, prophylactic anti-D is recommended. However, proper characterization of an *D* variant is essential to consider it differently as donor or patient, which is not possible serologically. Sandler et al suggested that the laboratory should stop giving report such as "**Serological weak D type**" and instead try to characterize *D* variant samples using molecular techniques (17). This practice will help in managing *D* variants in women and donors. Also, D variants like DELs, which are not detected serologically, can be genotyped to report correct RhD status.

Many D variant cells are difficult to characterize serologically due to variable nature of reactivity with monoclonal anti–Ds**.** In suspected D variant cases molecular biology can provide more objective based methods for correct RhD typing**.**

B) Molecular typing:

RHD gene, encoding D antigen is located on chromosome 1, in the tail to tail orientation with *RHCE* gene (encodes C,c, E and e antigens). Each of these two highly homologous (97%) genes contains 10 exons and is separated by 24 kb *TMEM50A* gene (Figure4). *RHD* gene is flanked by upstream and downstream rhesus boxes which are about 9000 bp homologous regions.

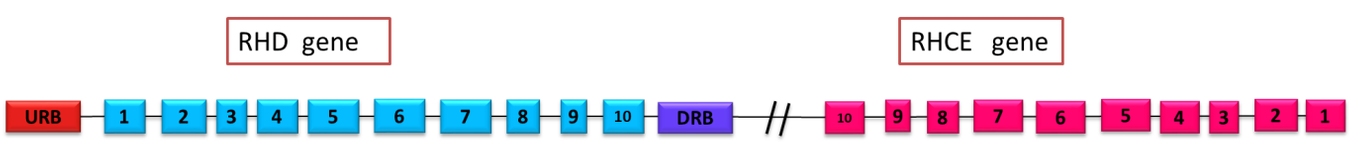

Figure 4: Chromosomal orientation of *RHD* and *RHCE* genes, Upstream rhesus box(URB) and Downstream rhesus box(DRB)

The factors such as proximity, opposite orientation and homologous nature enhance hybrid formation between these two genes. The common D variants formed as a result of hybridization include DVI type 1(*RHD-CE*(4-5)-*D*), DBT2 (*RHD-CE*(5-9)-*D*), DKK(*RHD-CE*(2-3)-*D*), DIIIc (*RHD-CE*(3)-*D*) etc. The RhCE variants like D--(*RHCE-D*(3-9)-*CE*), Dc-(*RHCE-D*(4-9)-*CE*, Dc-(*RHCE–D*(4–10)–*CE)* are also an outcome of a recombination event between *RHD* and *RHCE* genes. There are many point mutations within *RHD* gene which form *D* variants like DIII type 4(c.186G>T; c.410C>T; c.455A>C), DV type 3(c.667T>G; c.676G>C; c.697G>C; c.712G>A), DFV (c.667T>G), DAR1(weak D 4.2) (c.602C>G; c.667T>G; c.1025T>C)etc.

To overcome the limitations of serology, attempts were made to introduce molecular typing of RhD protein in routine blood banking. DNA typing by PCR requires sample preparation, nucleic acid extraction, and amplification of the desired diagnostic DNA fragment and specific detection of the amplicons**.** The large degree of diversity between D positive and D negative phenotypes complicate the issue of DNA based testing and has resulted in a gradual evolution of PCR based test. Based on the need and the accessibility of resources, the *D* typing method can be selected. Some of the useful genotyping techniques are as follows:

SSP-PCR

Nucleotide differences between *RHD* and *RHCE* have been used in D typing assay**.** Sequence-specific primers-PCR (SSP-PCR) is a widely used method to determine single nucleotide variants (SNVs). A perfectly matched primer pair can bind to the DNA and carry out efficient amplification. Allelic changes within the primer-binding site cannot allow attachment of the primers. Lack of PCR products denotes the change at the allelic site. Utilization of SSP-PCR to screen *RHD* specific region initiated in the year 1993. The initial screening strategy involved targeting Intron 4 of the *RHD* gene-specific in D positive individuals. Bennett et al. (1993) developed a PCR-SSP technique for prenatal D typing to detect the presence of *RHD* exon10 in the amniotic fluid and chorionic-villus sample (CVS)(18). Various D variants are formed due to point mutation like DAU-0(1136C>T), weak D type 1(809T>G), weak D type 10 (1177T>C), weak D type101(62A>C), RHD(1227G>A), RHD(G212R), RHD(L337R) etc. Many commercial available kits use SSP-PCR to type several D variants. For example, the BAGene (BAG Health Care GmbH, Germany) SSP-PCR typing kit contains Allele-specific primers, internal control primers and nucleotides coated strip tubes. By adding DNA, Taq polymerase and reaction buffer, one can type DNA for the presence of various partial D variants like DII, DIII, DIV, DV, DVI, DVII, DAU, DBT, DFR, DHMi, DHMii, DNB and DHAR (Rh33and weak D types including 1, 2, 3, 4.0/4.1, 4.2, 5, 11, 15 and 17.

PCR-RFLP

The restriction fragment length polymorphism (RFLP) technique utilizes the ability of a restriction enzyme to recognize and cut specific sequences within a stretch of DNA. In this method, a fragment of DNA is amplified using PCR-SSP. The product is then treated with a restriction enzyme, which forms different fragment based on the number of restriction sites. Allelic changes can alter a restriction site causing fragment length altered from wild type. This method is widely used to detect a hybrid box formed during *RHD* deletion. Wagner et al 1999 developed an RFLP-PCR for detection and classification of D variants like weak D types 1, 3, 5 & 6(19). PCR-RFLP developed by Wagner and Flegel(2000) use Pst 1 restriction enzyme which forms five, four and three fragments in *RHD* positive(hetero), *RHD* negative, *RHD* positive(homo) samples respectively(20). This method

predicts the *RHD* genotype of a child of a D negative mother by determining the zygosity of her D positive husband. Another study done by Lin et al (2003) utilized RFLP-PCR to screen seven weak D phenotypes (21). In this method, Due to point mutations, abnormal exon 1 and 6 fragments were generated after digestion with restriction enzymes.

Multiplex PCR

In a multiplex PCR assay, more than one target sequence can be amplified by using many primer pairs in a single test run using a common reaction mixture. Targeting various regions in a single tube is a challenging task. Annealing temperature, sensitivity and specificity of each primer must be thoroughly considered during primer designing. However once optimized, multiplex-PCR is found to be cost-effective. After multiplex PCR, products are visualized on agarose gels and differentiated by their different fragment sizes. Pope et al.(1995) combined multiple primers used in SSP-PCR to developed an *RHD* multiplex PCR (22). These primers can amplify *RHD* exon 7, a region between exon 4 & 5 and exon 10 in a single tube. Later attempts were made to amplify many *RHD* exons simultaneously. Maaskant-van Wijk, et al standardized multiplex PCR to amplify six *RHD* exons i.e. 3, 4, 5, 6, 7, and 9(23). Targeting multiple regions reduces the chance of false-negative results. As many *RHD* variants involve the formation of *RHD*-CE-D hybrids, targeting multiple exons helps to detect these variants.

Real-time PCR

The real-time PCR system is an advanced and sensitive version of classical PCR where amplification and copy number can be measured due to emission of fluorescence as the reaction progresses in real-time. It is carried out in a specialized thermal cycler where each sample is illuminated with a specific wavelength of light, which excites the fluorophores. The emitted fluorescence is detected using specialized filters. Real-time PCRs have wide-scale application in SNP genotyping. The advantage of this technique is that the relatively small amount of DNA can be rapidly and accurately reproduced and does not require post PCR processing in contrast to classical PCRs. Though probe-based detection is costly, it detects amplification of a specific region of interest. Real-time PCR has been implicated in the determination of *RHD* zygosity and noninvasive prenatal D typing. Fichou et al used Tm-shift assay to screen the presence of the most common mutations in the Caucasian population i.e weak *D* type 1, 2 and 3(24).

QMPSF

Quantitative multiplex polymerase chain reaction of short fluorescent fragments (QMPSF) is a semiquantitative method based on a simultaneous amplification by PCR (multiplex PCR) of short exonic fragments in a single tube using dye-labeled primers. Developed by Yann Fichou and Claude

Férec (2015), QMPSF is an invaluable technique that determines copy number variance of *RHD* and *RHCE* genes (25). *RHD* and *RHCE* genes share 96% homology, which promotes recombination between these genes. The crossing over causes the exchange of DNA fragment of one gene with another. In QMPSF, all 10 exons of *RHD* and *RHCE* are screened using sequence-specific primers in two different tubes. A FAM-labelled universal primer consists of λgt11 genetic sequence is used to label the products. These labelled products can be visualized in a sequencer using Liz-500 as a size standard. Based on the height and area of the peak, copy number of each *RHD* and *RHCE* exons can be calculated. This technique is especially suitable to detect variants formed due to hybridization (rearrangement of exons between *RHD* and *RHCE* genes). Recently novel duplication mechanism giving rise to weak D type 150, a predominant D variant in Indians, was characterized with the help of this technique (5).

RHD Sequencing

Developed by Sanger et al in 1977, DNA sequencing provides the nucleotide sequence of a DNA fragment under investigation. This method is especially useful to detect unknown variations within a span of DNA fragments. DNA sequencing involves in-vitro DNA polymerization using chain-terminating dye-labelled dideoxynucleotides (ddNTPs). Fluorophore labelled ddNTPs are added to the reaction along with deoxynucleotide triphosphates (dNTPs). The binding of ddNTP terminates the polymerization. Thus based on the position of nucleotide insertion, various fragments are generated which can determine by capillary electrophoresis. Wagner et al., 1999 developed a sequencing strategy to determine the sequence of all 10 *RHD* exons(26). With the help of this method, they could identify and characterize D variants as weak D type 1 to type 16. Later Okuda et al developed a sequencing strategy to amplify *RHD* and *RHCE* introns (27). *RHD* sequencing is very useful in determining various SNPs and short InDels.

Next-generation sequencing (NGS)

NGS is massively parallel, sequencing technique which sequences millions of fragments simultaneously per run and has been popularly used. Next-generation sequencing involves three basic steps: library preparation, sequencing, and data analysis. NGS requires sophisticated bioinformatics systems, fast data processing and large data storage capabilities, which can be costly. Tounsi et al (2018) carried out complete sequencing of the *RHD* gene by NGS using long-range PCR amplification overlapping entire *RHD* introns and exons(28). Using NGS they could detect various SNPs within intronic regions and linked their presence with R_1, R_2, R_0 and R_Z haplotypes. The targeted exome sequencing approach of Schoeman et al (2018) determined a novel *RHD*:c.452G>A allele (29). As the *RHD* gene is highly polymorphic in nature, Tammi et al (2020) have recommended the utilization of NGS for complex cases of *RHD* genotyping as compared to traditional PCR based methods (30).

NIIH experience:

Department of transfusion medicine, ICMR-NIIH has been involved in research related to the Rh blood group system for more than five decades. Rh clinic was established for D negative antenatal women since the 1970s. In 1995, Intrauterine transfusion facilities were created with help of Wadia maternity hospital to save the foetus from severe Rh-HDN. The tests including D typing, extended typing for common Rh antigens, Rh antibody titres, husband's blood group, zygosity, postnatal investigation of baby etc which have been performed for many years. Molecular testing for D antigen and identification of D variants was also initiated. Kulkarni et al, 2006 standardised *RHD*-PCR method using DNA from chorionic villus sample for prenatal diagnosis (31). A simple serological diagnostic strategy was developed to identify Indian *D* variants using different epitope specific anti-Ds. For molecular screening of the *RHD* gene, various methods like SSP-PCR, Real-time, Sanger's sequencing have been standardized. A very useful molecular technique called QMPSF was standardized to determine copy number variation within the *RHD* gene. By using this technique, we discovered the novel genetic mechanism giving rise to predominant Indian *D* variants (weak D 150). Figure 5 shows the timeline of various milestones achieved by ICMR-NIIH on their work on D variants. An indigenous multiplex PCR was designed to identify D variants by targeting exon 5, 10 and weak D type 150 allele. This screening technique now is being used routinely to determine the molecular background of D variants.(4).

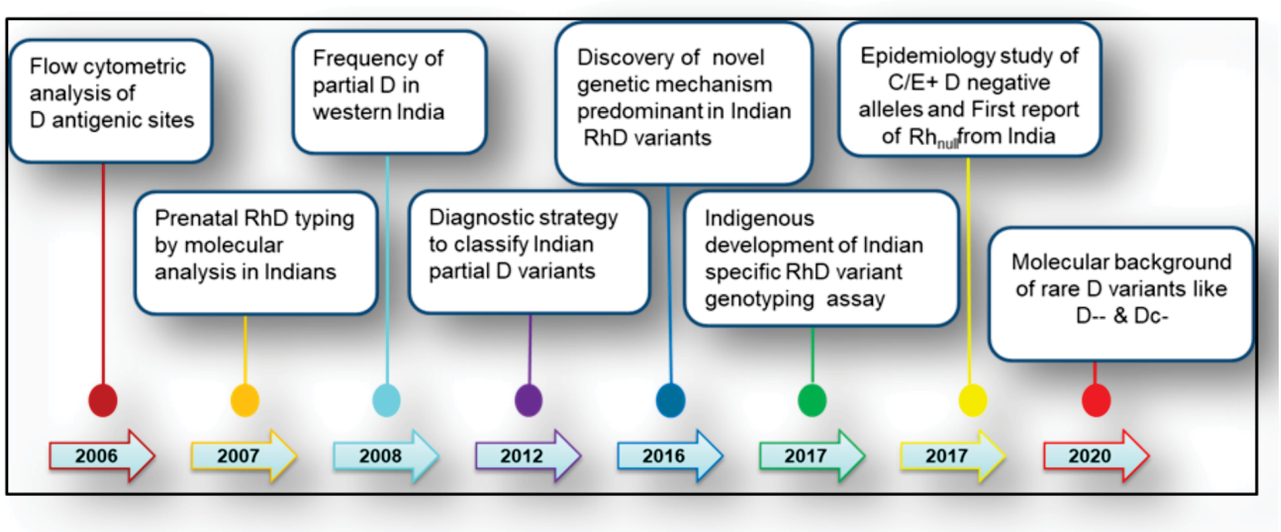

Figure 5: Milestones achieved by ICMR-NIIH in identification and molecular characterization of various RhD variants

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RhD टाइपिंग के लिए उपयोग की जाने वाली सीरोलॉजिकल एवं आणविक तकनीक और D वेरिएंट की पहचान में उनकी भूमिका

गरीमा मिश्रा

अनुवाद : सोमप्रकाश धनगर

Rh, एक प्रोटीन आधारित ब्लड ग्रुप सिस्टम है, जोकि मुख्यतः D, C, c , E और e प्रोटीन की उपस्थिति के आधार पर बनाया गया है। इनमें से RhD प्रोटीन इंसानो में सबसे अधिक मात्रा में उपस्थिति होती हैं। परंतु इसकी आवृत्ति संजातीय समूह में अलग-अलग पाई गई हैं, जैसे कि 85% युरोपियन में, 99% ईस्ट एशिया और 95% भारतीयों में यह पाई गई है। साथ ही RhD के विभिन्न प्रकारों के कारण 0.2 से लेकर 1% तक लोगों में RhD की उपस्थिति की सही स्थिति अभी तक ज्ञात नहीं है। RhD प्रोटीन को मुख्य रूप से तीन समूह में बांटा गया है- Weak D, Partial D और Del। यह वर्गीकरण RhD प्रोटीन की RBCs के ऊपर उपस्थित मात्रा एवं गुणवत्ता के आधार पर किया गया है। RhD प्रोटीन की अनुपस्थिति कई कारणों जैसे RHD जीन का विलोपन, हायब्रिडाइजेसन एवं सिंगल nucleotide म्युटेशन पर निर्भर करती है। RhD प्रोटीन की उपस्थिति चिकित्सीय रूप से महत्वपूर्ण है, क्योंकि यह भ्रूण में हिमॉलिटिक बीमारी के लिए उत्तरदायी होती है। किसी भी जनसमूह में उचित RhD टाइपिंग करने के लिए विस्तृत सेरोलॉजिकल एवं अनुवांशिक जाँच करना आवश्यक होता है। क्योंकि anti-D प्रतिजन के कारण होने वाला एलोइम्यूनाइजेशन RhD प्रोटीन के प्रकार पर निर्भर करता है। सेरोलॉजिकल टाइपिंग हिमइग्लूटीनेशन के सिद्धांत पर कार्य करती हैं, जिससे RhD प्रतिजन anti-Dअभिकर्मक से क्रिया कर रक्तकोशिकाओं के सुक्ष्म गुच्छे बना देता है। और इन अभिकर्मकों के आधार पर इस जांच को दो भागों में बांटा गया है- डायरेक्ट एवं इन- डायरेक्ट इग्लूटीनेशन जाँच। परंपरागत ट्यूब तकनीक, कॉलम आधारित तकनीक एवं तरल अवस्था आधारित माइक्रोप्लेट तकनीक आसान एवं कम लागत वाली विधि होने के कारण व्यापक रूप से उपयोग में लाई जाती हैं। साथ ही अन्य विधियां जैसे सतह प्लाज्मा रिजोनेंस एवं फ्लुरोसेंट सूक्ष्मदर्शी आधारित तकनीक भी उपयोग में लाई जाती हैं। इसके अलावा कुछ RhD प्रोटीन जैसे Del जो की बहुत कम मात्रा में होती हैं, को केवल एडसोर्पसन एलुशन तकनीक से ही जांच कर सकते हैं। हालांकि इस की आवृत्ति कम होने के कारण यह विधि ज्यादा उपयोग में नहीं लायी जाती है। RhD प्रोटीन की जांच उचित विधि से करना आवश्यक होता है। अगर ऐसा

नहीं किया जाए तो आकलन गलत होने की आशंका होती है।इसलिए कम से कम दो व्यवसायिक मोनोक्लोनल anti-D, जो कि दो अलग-अलग कंपनी द्वारा बनाए गए हो या अलग-अलग बैच के एक ही कंपनी द्वारा बनाए गए हो, का उपयोग कर जांच करना उचित होता है। फ्लो- साइटोमेट्री भी RhD प्रोटीन की मात्रा एवं इसकी उपस्थिति तथा Weak एवं Partial D में विभाजन हेतु उपयोग की जाती है। इन कमियों को पूरा करने के साथ ही ब्लड बैंक में RhD टाइपिंग के लिए अनुवांशिक जांच उपलब्ध कराने हेतु प्रयास किया जा रहा है। जिसके माध्यम से रक्त आधान संबंधी बीमारियों पर रोक एवं उनसे बचाव किया जा सकेगा। इस अनुवांशिक जांच के लिए विभिन्न विधियां जैसे SSP-PCR, PCR -RFLP, Multiplex PCR, Real - Time PCR एवं सीक्वेंसिंग उपयोग में लायी जा रही हैं। इनमें से QMPSF सबसे महत्वपूर्ण विधि है, जो कि RHD जीन की मात्रा (कॉपीनंबर) एवं युग्मनजता (zygosity) की जानकारी देती है।

ICMR-NIIH का ट्रांसफ्यूजन मेडिसिन विभाग कई दशकों से RhD टाइपिंग के लिए रक्त नमूनों का सेरोलॉजिकल एवं अनुवांशिक सत्यापन करने में अपनी महत्वपूर्ण भूमिका निभा रहा है। विभाग में प्राप्त सभी RhD नेगेटिव एवं Weak D प्रोटीन वाले सैंपल को एपिटोप विशेष मोनोक्लोनल anti-Ds पैनल के साथ जांच की जाती है। इसके पश्चात Weak D डी प्रोटीन वाले नमूने को अनुवांशिक विधि की मदद से सत्यापन किया जाता है। हाल ही में ICMR-NIIH के ट्रांसफ्यूजन मेडिसिन विभाग द्वारा एक नवीन RhD वेरिएंट, weak D - 150 की खोज की है, जो कि RHD जीन के एक्सॉन 3 के दोहरीकरण के कारण उत्पन्न होता है, जो कि लगभग 66% भारतीयों में पाया गया है। इस तरह के परिणामों के आधार पर RhD टाइपिंग के लिए एक्सॉन 5, 10 एवं दोहरीकृत भाग के सत्यापन हेतु एक Multiplex- PCR विधि विकसित की गई है, जो कि अब इस विभाग में नियमित रूप से प्रयोग में लाई जा रही हैं।

Mrs Anjali Nandgaonkar, Lab Assistant Farwell on 30th Sept, 2020

Mr Milind Dolas, Technical officer Farwell on 30th December, 2020

Vigilance oath taken by Scientist & staff on 30th October, 2020

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