# Advances in Blood Group Genotyping

Maria Rios*

The success of a blood transfusion event relies accurate blood typing. The importance of blood typing importance increases with the number of transfusions and consequent alloimmunization. Hemagglutination has been the assay of choice for blood typing because it is simple and does not require sophisticated technical skills or equipment. However, its interpretation is subjective because it depends on the quality of anti-sera. Extended red cell typing of transfusion dependent patients who have developed allo or autoantibodies remains a difficult laboratory problem.. Transfusion dependent patients are defined as those who require frequent transfusion throughout their lives. Among them are patients with aplastic anemia, some with myelodysplasia who are not eligible for stem cell transplantation, thalassemia, selected patients with sickle cell anemia who are managed with transfusion therapy, and patients with autoimmune hemolytic. Cell typing of sensitized patients is essential confirm the identity of suspected alloantibodies, and to facilitate the identification of antibodies that may develop in the future. In some occasions, typing of a heavily transfused patients is difficult because it is difficult to distinguish between donor and patient cells. Typing of sensitized patients with IgG antibodies is also difficult because the presence of this immunoglobulin on patient's red blood cells complicates the anti-globulin test. Typing transfused patients with sickle cell disorders by hemagglutination is relatively simple since allogeneic cells can be lysed with hypotonic solutions. In other cases, however, typing depends on the separation of the patient's reticulocytes and young red cells by differential centrifugation. These techniques are described in the AABB Technical Manual. Differential centrifugation may be impossible if the patient has been heavily transfused or has a low reticulocyte count. An alternative method to directly type reticulocytes utilizes two-color staining flow cytometric analysis[1]. Classically, patients with autoimmune hemolytic anemia are typed with IgG antisera following elution of the autoantibody from the cell surface. Unfortunately, elution techniques may inactivate Kell antigens, and treatment of cells with chloroquine may weaken Rh antigens

## The genetic basis of blood group polymorphisms

The great advance in molecular techniques led to the identification of the genetic basis for blood group antigens. The genetic mechanisms[2] involved in the generation of blood group polymorphisms are:

Point mutation, i.e., substitution of a single nucleotide in the coding region of a DNA strand can lead to the following consequences:

(1) no alteration in the amino acid specified by the codon (silent mutation).

(2) substitution in the identity of the encoded amino acid (missense mutation).

(3) conversion of a codon specifying an amino acid into one coding for termination of translation (nonsense mutation i.e. stop codon).

The majority of the blood group antithetical antigens arise from missense mutations[3-5]. Nonsense mutations are associated with null phenotypes of Duffy[6], Colton[7].

Deletion - loss of a single nucleotide or segment of DNA. A deletion of nucleotides in multiples of three, if in-frame, will result in the absence of one or more amino acid(s) and, most likely will generate a protein with different characteristics. A deletion of three nucleotides out of frame or in multiples of anything other than three (1, 2, 4, etc.) will induce a shift in the correct reading frame and an inactive mutant protein. Some blood group null phenotypes are generated from deletions of nucleotide(s), exon(s) or gene(s), e.g.: Jk(a–b–), U–, D–[7-11]

* *New York Blood Center*

Insertion – of a single nucleotide or segment of DNA. An insertion can be in-frame or can cause a frame shift. Some of the blood group null phenotypes that arise from this mechanism, e.g.: Co(a–b–)[7].

Altered splicing events - The outsplicing of introns is a precise process [2]. Specific motifs at the 5' and 3' ends of the introns are required and sometimes the 3' end of the exons is involved: the 5' intronic donor splice site is gt while the 3' intronic acceptor sequence is ag. A change in a single nucleotide in these motifs can alter the splicing to induce such events as exon skipping, for example the Jk(a–b–) phenotype[8].

There are also gross chromosomal alterations associated with blood groups[11-13] including:

Chromosomal translocation - transfer of a segment of one chromosome to another, non-homologous chromosome. Examples of blood groups that have been altered by this mechanism are A, B, D, H, M and N.

Single crossover - reciprocal exchange of part of a gene on one chromosome by part of a gene on the partner chromosome. In blood group systems, intergenic crossing over occurs in the MNS, Rh and Ch/Rg systems as they are encoded by two homologous genes adjacent on one chromosome.

Gene conversion and other rearrangements - heteroduplex DNA are formed as a consequence of pairing and recombination between non-identical alleles on the two chromosomes. In blood group systems, gene conversion has been described in Lu, MNS and Rh. Other rearrangements include complex hybrids in which the underlying mechanism has not yet been determined.

## DNA typing methodology

Once the molecular basis of an antigen is known, one can genotype the specific antigen by testing DNA obtained from nucleated cell of any source. These DNA typing technologies are based on PCR amplification of sequences of the genes of interest and identification of the gene alleles.

DNA – Cellular DNA suitable for the performance of molecular analysis by PCR amplification must be obtained. There are several commercially available kits for DNA isolation. The DNA sample should be analyzed for quantity and quality. Poor or absent amplification can be observed if the DNA quality is poor and the fragment to be amplified is 400bp or longer. The ideal amount for amplification of blood group genes ranges between 50 and 200 ng.

PCR protocols - There are several PCR amplification approaches that can be used for the determination of blood group polymorphisms. The most common are post-amplification analysis by restriction fragment length polymorphism (PCR-RFLP) and allele specific primers for PCR amplification (AS-PCR).

PCR-RFLP analysis is used for the identification of alleles that differ from each other by a nucleotide(s) substitution(s) that is (are) associated with the generation or ablation of restriction enzyme sites. Fortunately, this situation occurs with a large number of antigen systems. In the PCR-RFLP format a set of primers flanking the polymorphic DNA segment of interest is used for DNA amplification. After amplification the products are digested by the enzyme and electrophoresed in agarose or acrylamide gel.

AS-PCR is conceptually simpler than PCR-RFLP. In this technique, the 3’ end of one of the primers selected for amplification of the target sequence is specific for one of the alternate alleles, and the presence or absence of the allele is determined by the presence or absence of PCR product. AS-PCR is rapid and simple to perform when alleles differ in several nucleotides. It is difficult to distinguish alleles that by only one nucleotide. AS-PCR requires an internal control to assure assay efficacy. AS-PCR is used primarily with complex gene differences or when single point mutations do not result in generation or ablation of a restriction endonuclease site.

## Cell typing choice: To Genotype or To Phenotype

The hemagglutination assay is simple, inexpensive and, when done correctly, has appropriate specificity and sensitivity for the clinical care of the vast majority of patients. However, this assay is limited in some critical situations:

Identification of a fetus at risk of hemolytic disease of the newborn (HDN). DNA can be prepared from cells obtained by conventional invasive techniques

such as amniocentesis, or chorionic villus sampling, or cells obtained by non-invasive procedures such as trophoblasts collected by transcervical sampling[14-16] or from fetal erythroblasts from the maternal circulation[17,18]. Molecular genotyping should be considered when the mother's serum contains an alloantibody that has been associated with HDN and the father is heterozygous for the corresponding antigen or is not available for testing. The approach to molecular genotyping should err on the side of caution and include the detection in both fetus and mother, of gene or part of a gene whose product is not expressed on the RBC membrane but is expressed elsewhere (e.g. Fy(b–) due to GATA mutation). An important practical consideration is to determine whether the mother was the recipient of artificial insemination, *in vitro* fertilization, or whether she is a surrogate mother.

Determination of blood group alleles in multi-transfused patients – In recently transfused patients the transfused RBCs interfere with hemagglutination. Genotyping for blood group alleles can be performed on DNA prepared from white blood cells (WBCs) obtained from urine sediment or buccal epithelial cells[19], all of which are non-invasive procedures. However, before interpreting results, it is important to obtain a medical history to ascertain whether the patient has been transplanted with allogeneic stem cells obtained from bone marrow, peripheral blood or placental blood, or is known to be a chimera. In these situations, the genotype result obtained with DNA prepared from peripheral blood leukocytes is likely to be different than that obtained from DNA from other tissues. Our data show that donor leukocytes, remaining after transfusion of filtered RBC components, do not interfere with the PCR reactions under the stringency conditions we employed[20]. However, it should be noted that contamination with allogeneic cells is not uncommon in the clinical laboratory[21] and that transfused leukocytes may survive a long time in trauma patients who received 8 to 10 units of relatively fresh blood (microchimerism) [22].

Determination of blood group alleles when the patient's RBCs are coated with immunoglobulin - Molecular genotyping can be useful when the patient's RBCs are coated with IgG and appropriate direct agglutinating antibodies are not available and chemical treatment of the RBCs does not remove sufficient IgG to give confident results. This is especially true when only weakly reactive antibodies are available. This approach is also useful when the chemical treatment inactivates the antigen.

Resolution of weak A, B and D typing discrepancies - In case of discrepancy in ABO or D typing (especially of weak antigens in donors). A proportion of blood donors and patients who historically have been typed as group O are now being typed as group A or group B because the new reagents containing monoclonal antibodies detect small amounts of the immunodominant carbohydrate responsible for A or B specificity. In the past, RBC products from such donors (i.e., having weak A or B antigens) have been transfused to group O patients without untoward sequelae. However, typing results that differ from the historical record often result in time-consuming analyses such as absorption and elution studies to determine the true ABO group of the person. The molecular basis of many of the weak subgroups of A and B is known and reflect altered transferase genes that can be detected by PCR[23-27].

A similar situation occurs with the D antigen of the Rh blood group system. Some blood donors who were typed as D-negative are now being typed as D-positive because the newer reagents contain monoclonal antibodies that are capable of detecting small specific parts of the D antigen. The molecular basis of numerous D variants has been determined and the knowledge can be used to identify the genes encoding altered RhD protein in these people. Furthermore, in transfusion management it would be helpful to determine which patients typed phenotypically as Fy(b–) can be transfused with Fy(b+) RBCs.

Technical comments - Missense mutations associated with blood group antigens that give rise to a gain or loss of a restriction enzyme site are particularly useful in the clinical laboratory. This is especially true when they produce a unique banding pattern with both alleles because a distinct pattern is obtained thereby eliminating the need for an internal control to verify that each test has been performed correctly[28]. Since the quantity and quality of DNA in clinical samples is often less than ideal, it is recommended that the selected primers generate relatively short PCR amplified products

prior to RFLP analysis. It is also recommended that known homozygotes and heterozygotes are tested in parallel. Detailed technical considerations have been reviewed elsewhere[3].

Discordance between genotype and phenotype - There are many situations where the genotype and phenotype will not correlate:

*Medical reasons*- patients who received a transplant of allogeneic stem cells from cord blood, bone marrow, or peripheral blood, mothers who were artificially inseminated or are surrogate mothers. Although it is obvious that in such cases the genotype will differ from the father/mother, this information is not always provided. In general, there should be no concern about the results obtained from the peripheral blood of chronically transfused recipients because the patient's leukocytes predominate[20,29].

*Molecular reasons* - point mutation in a regulatory element of a gene thereby impairing transcription, mutation leading to stop codon (nonsense mutation), a mutation in splicing sites of a gene, a premature stop codon, the absence of an interacting protein, and a decreased amount of protein in the RBC membrane[13]. In all cases PCR-based assays would predict a certain antigen profile and yet the gene product would not be expressed in the RBC membrane. There are other examples where an apparently normal gene is present and yet there is no RBC surface expression e.g., D-negative, $K_0$, Jk(a-b-). The reason for this phenomenon has not been determined. Other molecular events, such as crossovers and other gene rearrangements can also lead to situations where the genotype and phenotype will not agree. In this situation, depending on their location, the primers may or may not anneal to the hybrid gene and thereby could give false negative or false positive genotyping results. This occurs when two homologous genes are juxtaposed on the same chromosome, e.g.: MNS and Rh. Many "modifying" genes influence antigen expression: $Wr^b$ antigen is encoded by the wild type of band 3[30] and is only expressed if amino acids 59 to 76 of glycophorin A (GPA) are present[31,32]; Kell system antigens require the presence of the Xk protein to be fully expressed[33]; Rh antigens (D, C, c, E, e) require the presence of the Rh associated glycoprotein (RhAG) for expression[34]; Gerbich blood group system antigens are poorly expressed in the absence of protein 4.1 because only a proportion of glycophorins C and D is held on the RBC membrane[35]. In addition, antigens in the Kidd and Lutheran blood group systems are weakened by the presumed presence of *In(JK)* and *In(LU)* respectively. These modifying genes have not been identified.

In summary, DNA genotyping offers the opportunity to reliably type transfusion dependent patients and thereby improve the safety of transfusion. Lastly, the molecular basis of most known RBC antigens was determined by analysis of a relatively small number of people with known antigen profiles and it is assumed that they can be extended to all populations. It should be noted that a much larger number of people from a variety of ethnic backgrounds need to be studied in order to establish more firmly the correlation between genotype and the blood group phenotype. Until such information is available, molecular genotyping will continue to be a valuable adjunct to hemagglutination and will not replace this tried and true RBC typing method.

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