Molecular testing for transfusion medicine

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Purpose of review

Molecular testing methods were introduced to the blood bank and transfusion medicine community more than a decade ago after cloning of the genes made genetic testing for blood groups, that is genotyping, possible. This review summarizes the progress made in the last decade in applying genotyping to prenatal practice and clinical transfusion medicine.

Recent findings

Assays that target allelic polymorphisms prevalent in all populations are reproducible and highly correlated with red blood cell phenotype. For some blood groups, assays that detect silencing mutations are also required for accurate typing, and for ABO and Rh, multiple regions of the genes must be sampled. Genotyping is a powerful adjunct to serologic testing and is superior for typing transfused patients, for D-zygosity determination, for noninvasive fetal typing, and for antigen-matching in sickle cell patients.

Summary

Implementation of molecular testing for transfusion medicine has been a conservative process and limited primarily to reference laboratory environments. With the development of high-throughput platforms, genotyping is poised to move into the mainstream, revolutionizing the provision of antigen-negative donor units. This will enable electronic selection of units antigen matched to recipients at multiple blood group loci, potentially eliminating alloimmunization and significantly improving transfusion outcomes.

Keywords

genotyping, molecular testing, prenatal testing, RHD zygosity, single nucleotide polymorphisms

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Abbreviations

FDAUS	Food and Drugs Administration
PCR	polymerase chain reaction
RBC	red blood cell
SCD	sickle cell disease
SNP	single nucleotide polymorphisms

Introduction

The age of genomics has enabled the application of DNA-based molecular methods to clinical laboratory diagnostic testing in the areas of genetics, hematopathology, inherited thrombosis, and infectious disease, to name a few. Molecular methods are also applicable to blood bank and transfusion service testing [1,2]. The rapid progress made in determining the genetic basis for blood group and platelet antigen polymorphisms, and the commercial development of polymerase chain reaction (PCR)-based technology, makes detection of blood group antigens by probing the gene now possible.

Many blood group antigens are the result of single nucleotide gene polymorphisms or SNPs (pronounced 'snips') inherited in a straightforward Mendelian manner. SNPs occur approximately every 300–500 base pairs (http://www.ornl.gov/sci/techresources/Human_Genome/faq/snps.shtml), and account for much of the diversity of the human genome. SNPs have become the target of testing strategies for genetic disease and diagnostics, and the possibility that they can be linked to disease risk factors, or to inter-individual variations in drug responses, has broadened the appeal and potential for SNP profiling. This has precipitated the development of high-throughput genotyping platforms that utilize microarray and chip technologies. Testing for blood group antigens in transfusion medicine is poised to benefit from this wave of new technology.

Molecular genotyping methods were introduced to the transfusion medicine community over a decade ago. Implementation of DNA-based methods in the blood bank environment benefits from the ability to validate a specific SNP/antigen association by testing a large number of samples with concurrent red blood cell (RBC) serologic and DNA testing. To date, molecular testing has been done primarily in the reference laboratory setting, as the first generation of blood group genotyping assays are time consuming and labor intensive, and discrepancies require serologic and genetic follow-up.

This has been a decade of discovery concerning the genetic variations that underlie blood group antigen expression in different ethnic groups. Although most blood group antigens are encoded by single SNPs, the genes responsible for expression of the antigens of the principal systems, ABO and Rh, are more complex. Over 100 different glycosyltransferase genes give rise to A, B, or O blood groups, including weak A and B subgroups,

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hybrids, and inactive O enzymes [3,4]. These complicate *ABO* genotyping. Similarly, there are well over 100 different *RHD* genes encoding proteins with single amino acid changes, or rearranged genes encoding hybrid proteins, and over 50 different *RHCE* genes with single or multiple amino acid changes. These numerous mutations are summarized on the blood group mutation database (BGMUT) and RhesusBase websites [4–6]. Which A and B subgroups, and which *RH* gene variants, are clinically important to transfusion practice is an active area of investigation. Clarification of the clinical significance of the numerous gene variants requires serology and genetics, and reflects the power of the combination of the two approaches.

Why molecular testing for transfusion medicine?

Isolation of the molecules that carry blood group antigens, followed by the cloning of the genes and the development of DNA sequencing and PCR, have all paved the way for application of genetic information to blood transfusion and prenatal practice. In the last two decades, genes for all but one of the 29 blood group systems have been identified, thus presenting an alternative approach to the determination of blood group 'typing', that is, that of determining the genotype.

Development of alternative methods to determine blood groups is not just of academic interest. There are situations in which the genotype is a superior, or the only, approach. For example, serologic RBC agglutination tests have limitations when patients are multiply transfused or have RBCs coated with immunoglobulin. In addition, agglutination tests depend on the availability of specific and potent reagents, but these are not available for many blood group antigens, such as Dombrock (Do^a, Do^b), Colton (Co^a,Co^b), etc., or for low-frequency antigens in the majority of blood group systems. Furthermore, discrepancies in serologic reactivity between different manufacturers' reagents can occur, complicating determination of the antigen status of RBCs. Genotyping can resolve these. Finally, D-zygosity testing and fetal typing from amniocytes or from cell-free fetal DNA present in maternal plasma can only be done by molecular testing.

When is determining the genotype superior to the phenotype?

Agglutination tests have been the gold standard for identification of blood group antigens for over 100 years, but there are some limitations.

Typing multiply transfused patients

In patients receiving chronic or massive transfusion, the presence of donor RBCs in the peripheral blood often makes RBC phenotyping by agglutination techniques inaccurate. Genotyping overcomes this limitation. PCR

assays for blood group genes avoid interference from donor-derived DNA by targeting and amplifying a region of the gene common to all alleles. This approach, in contrast to targeting and amplifying one specific allele, allows reliable blood group determination with DNA prepared from a blood sample collected after transfusion [7,8]. In transfusion-dependent patients who produce alloantibodies, an extended antigen profile is important to determine additional blood group antigens to which the patient can become sensitized.

Typing red blood cells with a positive direct antiglobulin test

In patients with autoimmune hemolytic anemia, or with RBCs coated with immunoglobulin in the absence of hemolysis, the presence of bound immunoglobulin G (IgG) often makes RBC phenotyping by the indirect antiglobulin test invalid. IgG removal techniques are not always effective and can destroy the antigen of interest. Genotyping offers an alternative approach.

Screening donor units when reagents are not available

Genotyping can be used to screen donor blood for transfusion when no reliable typing reagent is available. The Dombrock blood group polymorphism is an often cited example, and detection of a single SNP is robust and reproducible to type donors and patients for Do^a and Do^b [9,10]. These methods are not yet approved by the US Food and Drug Association (FDA) for labeling donor units, but they can be used to screen and select units for patients with these antibodies. As automated procedures result in faster throughput at lower cost, typing of blood donors by DNA-based assays will become more widespread, revolutionizing the provision of antigen-negative blood.

RHD zygosity testing

Serologic testing for RBC expression of D, C/c, and E/e can only predict the likelihood that a sample is homozygous (D/D) or heterozygous (D/−) for *RHD*. Molecular genotyping, however, enables zygosity to be determined by assaying for the presence of a recessive D-negative allele. In prenatal practice, paternal *RHD* zygosity testing is important to predict the fetal D status when the mother has anti-D. Several different genetic events cause a D-negative phenotype [11,12], and multiple assays must be done to accurately determine zygosity. These include detection of the region generated by deletion of *RHD*, and detection of the 37 base pair insert *RHD* pseudogene and the D-negative *RHD-CE-D* hybrid gene common in African Black ethnic groups [13,14]. If the father is *RHD* homozygous, the fetus will be D-positive, and monitoring of the pregnancy will be required. If the father is heterozygous, the D type of the fetus should be determined to prevent invasive and unnecessary testing.

Fetal typing

Genotyping is important in the prenatal setting to determine whether the fetus has inherited the paternal antigen to which the mother has a clinically significant antibody. If determined to be antigen negative, the fetus is not at risk for hemolytic disease of the fetus and newborn (HDFN) or neonatal alloimmune thrombocytopenia (NAIT), and the mother need not be aggressively monitored or receive immune modulating agents. To determine the antigen status, fetal DNA can be isolated from cells obtained by amniocentesis or chorionic villus sampling. Alternatively, the discovery that cell-free, fetal-derived DNA is present in maternal plasma or serum by approximately 5 weeks of gestation allows maternal plasma to be used as a source of fetal DNA [15]. This important observation enables noninvasive prenatal diagnosis, fetal sex determination, and testing for paternally inherited single gene disorders [16–18]. Fetal DNA in maternal plasma is derived from apoptotic syncytiotrophoblasts [19•], and increases in concentration with gestational age, but is rapidly cleared following delivery [15,17]. The latter observation indicates that, unlike fetal lymphocytes which can persist for years in maternal blood and skin [20,21], cell-free fetal DNA from previous pregnancies will not interfere with testing. When combined with sensitive real-time PCR methodology, isolation of fetal DNA from maternal plasma has been successfully applied to determine fetal D status. Several large-scale trials have now validated this approach [19•,22–25]. This has been particularly successful for D typing because the D-negative phenotype in the majority of samples is due to the lack of the RHD gene. Testing for the presence or absence of a gene is less demanding than testing for a single gene polymorphism or SNP. Theoretically, testing the maternal plasma for the presence of a fetal RHD gene could be used to eliminate the unnecessary administration of antepartum Rh immune globulin to the approximately 40% of D-negative women who are carrying a D-negative fetus. Rh immune globulin is not entirely risk free, and this approach would be cost-effective for some healthcare systems [19•]. The small quantity of cell-free fetal DNA present relative to maternal DNA, however, poses a challenge. Positive controls for isolation of sufficient fetal DNA are critical to validate negative results. Y chromosome markers are useful when the fetus is a male, but when the fetus is female, polymorphic paternal markers are needed [23,25]. The possibility of misinterpretation due to inheritance of rare or familial RHD inactivating mutations, or rearranged hybrid genes, can be prevented by testing samples from the parents. Standardization of protocols are still needed; however, it is likely that determination of fetal RHD with this noninvasive procedure will become routine clinical practice.

Fetal typing for the Kell blood group system and for the platelet HPA-1a (PL^A) antigen can also be important. For determination of the inheritance of these antigens encoded by SNPs, rather than by the presence or absence of the gene, a sample source that contains primarily fetal DNA, for example, amniocytes, is preferred.

Resolving reagent typing discrepancies

Serologic typing discrepancies can occur when testing RBCs with different reagent monoclonal antibodies. This occurs not infrequently with D typing, because the D status is complicated by the large number of different RHD genes, which can affect both the level of expression and, potentially, the structure and epitopes of the D antigen. There are more than 100 different RHD genes known, which include over 50 that encode different forms of weak D, approximately 40 that result in expression of partial D antigens, and several RHCE genes that encode D epitopes on the Rhce protein. The different monoclonal antibody clones present in manufacturers' reagents can react differently with these variant D antigens [26•]. Discrepancies occur because of differences in methods (tube tests, solid phase, gel, and automated analyzers), antibody clones, and the variability of D antigen expression. These can be resolved with genotyping.

More than 100 different ABO alleles have been reported [3,4]. Many different mutations cause reduced antigen expression characteristic of A and B subgroups, and some nondeletion O alleles express very weak A antigen. Discrepancies in ABO typing can result when antigen expression is depressed or weak to undetectable isoagglutinin activity is encountered. Similar to above, the different monoclonal antibody clones present in manufacturers' reagents can react differently with A and B subgroup RBCs. Genotyping is also a useful tool for the resolution of ABO typing discrepancies and is especially valuable for distinguishing acquired phenotypes from inherited ones. In conclusion, genotyping is extremely useful to resolve apparent ABO and Rh typing discrepancies. This is particularly important for blood donors, when discrepancies often involve recall of the blood product and must be reported to the FDA.

Distinction between weak D and partial D

As mentioned above, altered expression of the D antigen is not uncommon. Weak D have single amino acid changes that affect the quantity of protein in the membrane [27], resulting in a reduced number of D antigen sites on the RBCs. Partial D have amino acid changes that alter D epitopes, or often are hybrid proteins with portions of RhD joined to portions of RhCE (summarized in [6]) The distinction between weak D and partial D phenotypes is of clinical importance because the latter make anti-D. Routine serologic typing reagents cannot

distinguish between these RBCs, however, genotyping strategies that sample multiple regions of *RHD* can discriminate weak D and partial D phenotypes.

Detecting patients at risk for production of antibodies to high incidence antigens

Alloimmunization is a serious complication of chronic transfusion, particularly in patients with sickle cell disease (SCD) requiring long-term transfusion support. Many transfusion programs attempt to prevent or reduce the risk and incidence of alloantibody production in SCD patients by transfusing RBCs that are antigen matched for D, C, E, and Kell. Variant *RHD* and *RHce* genes are common in African Blacks and individuals of mixed ethnic backgrounds [28–30]. The prevalence of *RH* alleles that encode altered D, C, and e antigens in this patient group explains why some SCD patients become immunized to Rh, despite conventional Rh antigen matching. These antibodies often have complex, high incidence Rh specificities, and it can be difficult or impossible to find compatible units. Importantly, genotyping can identify those patients who are homozygous for variant RH alleles and at risk for production of alloantibodies to high incidence Rh antigens.

Conclusion

Molecular testing methods were introduced to the blood bank and transfusion medicine community more than a decade ago. Since then, efforts have focused on documenting that the blood group genotype reflects antigen expression on the red cell, that is, the phenotype, for many different blood group systems and in different ethnic groups. Significant progress has now been made in validating the gene targets and investigating and explaining discrepancies. Assays for blood group antigens encoded by single SNPs are highly reproducible and correlate with RBC phenotype. Genotyping for the two most important blood group systems, ABO and Rh, are more challenging because of the many different mutations responsible for weak subgroups of A and B, and inactive O, and because of the numerous variant and hybrid *RH* genes. Multiple regions of the genes must be sampled and complex algorithms applied for interpretation. Testing platforms that utilized bead or microarray technology, however, can readily sample multiple regions of the genes and apply automated multifaceted algorithms for accurate interpretations. This holds promise for eventual successful ABO and Rh genotyping.

Although agglutination tests have been the mainstay for identification of blood group antigens for over 100 years, genotyping is superior to serologic testing in several situations. These include typing multiply transfused patients and screening donor and patients when antibody reagents are not available or in short supply. Determining paternal *RHD* zygosity to predict hemolytic disease of the newborn, and typing the fetus from amniocytes or from the maternal plasma can only be done by genotyping. *RH* genotyping can identify SCD patients who are homozygous for variant alleles and at risk for production of alloantibodies to high incidence Rh antigens. When partnered with *RH* genotyping of donors, this would have a positive impact by reducing alloimmunization in SCD and would optimize the use of minority donations, as not all SCD patients require blood from minority donors.

The first generation of blood group genotyping assays are cumbersome and time intensive, consisting of PCR-RFLP (restriction fragment length polymorphism) and PCR-AS (allele specific) assays that require post-PCR sample manipulation and gel electrophoresis to separate and analyze the pattern of fragments. The introduction of real-time PCR with automated fluorescence discrimination of alleles has the advantage of a single, closed-tube system, with medium throughput. Amplification of multiple blood group systems in one tube, that is multiplex PCR, expands this application. All these approaches are labor intensive and limit widespread application of genotyping to screen large numbers of samples.

Numerous recent publications detail experience with several different high-throughput systems to genotype multiple blood group antigens [31,32•,33•,34,35]. Blood group genotyping is poised to move into the mainstream to revolutionize the provision of antigen-negative donor units. The challenge for the next decade lies in integrating genotyping into the donor center, standardizing methods [36•], and obtaining FDA approval. The application of molecular genotyping to transfusion medicine practice will dramatically change blood bank testing by enabling electronic selection of donor units antigen matched for recipients at multiple blood group loci. This approach holds great promise to improve patient care and transfusion outcomes by potentially eliminating alloimmunization. In the process, this approach would also dramatically change antibody and reference laboratory workloads and potentially alter the focus of donor recruitment.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 500).

1   Reid ME. Applications of DNA-based assays in blood group antigen and antibody identification. Transfusion 2003; 43:1748–1757.

2   Reid ME, Lomas-Francis C. Molecular approaches to blood group identification. Curr Opin Hematol 2002; 9:152–159.

3   Chester MA, Olsson ML. The ABO blood group gene: a locus of considerable genetic diversity. Transfus Med Rev 2001; 15:177–200.

4   Blumenfeld OO, Patnaik SK. Allelic genes of blood group antigens: a source of human mutations and cSNPs documented in the Blood Group Antigen Gene Mutation Database. Hum Mutat 2004; 23:8–16.

5   Wagner FF, Flegel WA. The rhesus site. http://www.uniulm.de/~fwagner/RH/RB/. [Accessed 8 August 2006]

6   Reid ME, Lomas-Francis C. The blood group antigen facts book. 2nd ed. San Diego, CA: Academic Press; 2004.

7   Reid ME, Rios M, Powell VI, et al. DNA from blood samples can be used to genotype patients who have recently received a transfusion. Transfusion 2000; 40:48–53.

8   Wenk RE, Chiafari PA. DNA typing of recipient blood after massive transfusion. Transfusion 1997; 37:1108–1110.

9   Storry JR, Westhoff CM, Charles-Pierre D, et al. DNA analysis for donor screening of Dombrock blood group antigens. Immunohematol 2003; 19:73–76.

10 Rios M, Hue-Roye K, Lee AH, et al. DNA analysis for the Dombrock polymorphism. Transfusion 2001; 41:1143–1146.

11 Colin Y, Cherif-Zahar B, Le Van Kim C, et al. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. Blood 1991; 78:2747–2752.

12 Daniels G, Green C, Smart E. Differences between RhD-negative Africans and RhD-negative Europeans. Lancet 1997; 350:862–863.

13 Chiu RW, Murphy MF, Fidler C, et al. Determination of RhD zygosity: comparison of a double amplification refractory mutation system approach and a multiplex real-time quantitative PCR approach. Clin Chem 2001; 47:667–672.

14 Singleton BK, Green CA, Avent ND, et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the Rh D-negative blood group phenotype. Blood 2000; 95:12–18.

15 Lo YM, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997; 350:485–487.

16 Lo YM. Recent developments in fetal nucleic acids in maternal plasma: implications to noninvasive prenatal fetal blood group genotyping. Transfus Clin Biol 2006; 13:50–52.

17 Lo YM, Hjelm NM, Fidler C, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med 1998; 339:1734–1738.

18 Nelson M, Eagle C, Langshaw M, et al. Genotyping fetal DNA by noninvasive means: extraction from maternal plasma. Vox Sang 2001; 80:112–116.

19 Van der Schoot CE, Soussan AA, Koelewijn J, et al. Noninvasive antenatal
•   RHD typing. Transfus Clin Biol 2006; 13:53–57.
The authors summarize the recent large scale patient studies and the diagnostic accuracy of prenatal RHD genotyping on cell-free fetal DNA.

20 Artlett CM, Smith JB, Jimenez SA. Identification of fetal DNA and cells in skin lesions from women with systemic sclerosis. N Engl J Med 1998; 338:1186–1191.

21 Bianchi DW, Zickwolf GK, Weil GJ, et al. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA 1996; 93:705–708.

22 Gautier E, Benachi A, Giovangrandi Y, et al. Fetal RhD genotyping by maternal serum analysis: a two-year experience. Am J Obstet Gynecol 2005; 192:666–669.

23 Finning K, Martin P, Daniels G. A clinical service in the UK to predict fetal Rh (Rhesus) D blood group using free fetal DNA in maternal plasma. Ann N Y Acad Sci 2004; 1022:119–123.

24 Rouillac-Le Sciellour C, Puillandre P, Gillot R, et al. Large-scale prediagnosis study of fetal RHD genotyping by PCR on plasma DNA from RhD-negative pregnant women. Mol Diagn 2004; 8:23–31.

25 Bianchi DW, Avent ND, Costa JM, van der Schoot CE. Noninvasive prenatal diagnosis of fetal rhesus D: ready for prime(r) time. Obstet Gynecol 2005; 106:841–844.

26 Westhoff CM. Review: the Rh blood group D antigen: dominant, diverse, and
•   difficult. Immunohematol 2005; 21:155–163.
This review details the variant D antigens that cause D typing discrepancies with current FDA licensed reagents in the USA.

27 Wagner FF, Gassner C, Muller TH, et al. Molecular basis of weak D phenotypes. Blood 1999; 93:385–393.

28 Hemker MB, Ligthart PC, Berger L, et al. DAR, a new RhD variant involving exons 4, 5, and 7, often in linkage with ceAR, a new Rhce variant frequently found in African Blacks. Blood 1999; 94:4337–4342.

29 Daniels GL, Faas BH, Green CA, et al. The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. Transfusion 1998; 38:951–958.

30 Noizat-Pirenne F, Lee K, Pennec PY, et al. Rare RHCE phenotypes in black individuals of Afro-Caribbean origin: identification and transfusion safety. Blood 2002; 100:4223–4231.

31 Beiboer SH, Wieringa-Jelsma T, Maaskant-Van Wijk PA, et al. Rapid genotyping of blood group antigens by multiplex polymerase chain reaction and DNA microarray hybridization. Transfusion 2005; 45:667–679.

32 Montpetit A, Phillips MS, Mongrain I, et al. High-throughput molecular profiling
•   of blood donors for minor red blood cell and platelet antigens. Transfusion 2006; 46:841–848.
Simultaneous analysis of eight blood group antigen pairs and three allelic platelet antigens with GenomeLab SNPstream genotyping gave 97.3–100% concordance, depending on the blood group, and emphasizes the importance of the gene target.

33 Hashmi G, Shariff T, Seul M, et al. A flexible array format for large-scale, rapid
•   blood group DNA typing. Transfusion 2005; 45:680–688.
BeadChip analysis for 18 SNPs representing 36 blood group alleles were concordant with manual PCR-RFLP, PCR-AS, and serologic typing when performed.

34 Denomme GA, Van Oene M. High-throughput multiplex single-nucleotide polymorphism analysis for red cell and platelet antigen genotypes. Transfusion 2005; 45:660–666.

35 Petrik J. Microarray technology: the future of blood testing? Vox Sang 2001; 80:1–11.

36 Daniels G, van der Schoot CE, Olsson ML. Report of the First International
•   Workshop on molecular blood group genotyping. Vox Sang 2005; 88:136–142.
Established to develop international consensus and standards for molecular genetic technology for blood group typing.