REPORT

2007 Rock Øyen Symposium

This issue of *Immunohematology* contains a series of papers that formed the basis of a seminar held at the New York Blood Center in November 2007. The seminar, titled "Understanding Molecular Analysis for Prediction of Blood Groups," was the first annual meeting to honor the contributions of Ragnhild "Rock" Øyen and Carol Johnson to the field of immunohematology. Rock and Carol had much in common: both were technologists, scientists, educators, and friends who worked together as colleagues at NYBC for more than 30 years and published numerous papers, reviews, and textbook chapters. They loved the work and the people, epitomized good customer service and relations, and were well respected. Tragically, both died prematurely in 2007 after courageously fighting cancer. The seminar was sponsored by the Immunohematology Education Fund, whose purpose and mission is to encourage medical technologists to follow Rock and Carol's role model and embrace a career in immunohematology. Although managed and administered by the New York Blood Center, this fund supports technologist and SBB education across the country.

The first paper, "From DNA to Blood Groups," by Marion Reid, provides an overview of how blood groups are encoded by changes in DNA sequence. The second paper, "Principles of PCR-Based Assays," describes techniques used in a molecular biology laboratory to predict blood group antigens, and is contributed by Kim Hue-Roye and Sunitha Vege. This paper is followed by a review of how the results of DNA testing can be applied to donors, by Donna Strauss and Marion Reid, entitled "Value of DNA Testing for Donor Screening and Regulatory Issues," and a review by Christine Lomas-Francis and Helene DePalma entitled "DNA-Based Assays for Patient Testing: Their Application, Interpretation, and Correlation of Results." It is clear that DNA testing is an invaluable adjunct to hemagglutination, and scenarios in which DNA testing can be used to overcome long-standing limitations of hemagglutination are described. The final paper in the series explains how more precise matching of donor RBC components with patients may be possible and feasible at the DNA level, especially for certain patient populations, and is entitled "The Potential of Blood Group Genotyping for Transfusion Medicine Practice," by Connie Westhoff.

We think Rock Øyen and Carol Johnson would have enjoyed the seminar and would have marveled at the potential for DNA testing to enhance the provision of antigen-negative RBC components and the selection of compatible blood for transfusion. Indeed, we hope you also enjoy this "molecular journey" highlighting, in our opinion, the most significant new tool in the field since the discovery of antibodies and agglutination.

Connie M. Westhoff

Marion E. Reid

From DNA to blood groups

M.E. Reid

A blood group antigen is a protein or carbohydrate on the outer surface of a RBC. Portions of DNA are transcribed and translated into proteins. A protein-based blood group antigen is the direct product of a gene whereas a carbohydrate-based blood group antigen is an indirect product of a gene; the gene product is a glycosyltransferase that transfers a carbohydrate moiety to a protein, or to another carbohydrate to form a chain of sugars. This report gives a brief description of a gene, its processing from DNA through RNA to an amino acid sequence, and how changes in nucleotides give rise to blood group antigens. ***Immunohematology* 2008;24:166–169.**

Key Words: application of molecular testing for blood groups in transfusion medicine, blood group alleles, blood group antigens, DNA to protein, prediction of blood groups

A blood group antigen is a variant form of a protein or carbohydrate on the outer surface of a RBC that is identified when an immune response (alloantibody) is detected by hemagglutination in the serum of a transfused patient or pregnant woman. The astounding pace of growth in the field of molecular biology techniques and in the understanding of the molecular bases associated with most blood group antigens and phenotypes enables us to consider the prediction of blood group antigens using molecular approaches. Indeed, the knowledge is currently being applied to help resolve some long-standing clinical problems that cannot be resolved by classic hemagglutination.[1] This report reviews the processing of DNA and molecular events that can lead to a blood group antigen.

Blood group antigens are inherited, polymorphic, structural characteristics located on proteins, glycoproteins, or glycolipids on the exofacial surface of the RBC membrane. The classic method of testing for blood group antigens and antibodies is hemagglutination. This method is simple and, when done correctly, has a specificity and sensitivity that is appropriate for the clinical care of the vast majority of patients. Indeed, direct and indirect hemagglutination tests have served the transfusion community well for, respectively, more than 100 and more than 50 years. However, in some aspects, hemagglutination has limitations. For example, it gives only an indirect measure of the potential complications in an at-risk pregnancy, it cannot precisely indicate *RHD* zygosity in people with D+ RBCs, it cannot be relied on to type some recently transfused patients, and it requires the availability of specific reliable antisera. The characterization of genes and determination of the molecular bases of antigens and phenotypes has made it possible to use the PCR[2,3] to amplify the precise areas of DNA of interest to detect alleles encoding blood groups and thereby predict the antigen type of a person.

From DNA to Blood Groups

The Language of Genes

DNA is a nucleic acid composed of nucleotide bases, a sugar (deoxyribose), and phosphate groups. The nucleotide bases are purines (adenine [A] and guanine [G]) and pyrimidine (thymine [T] and cytosine [C]). The language of genes is far simpler than the English language. Compare four letters in DNA or RNA (C, G, A, and T [T in DNA is replaced by U in RNA]) with 26 letters of the English alphabet. These four letters (nucleotides) form "words" (called codons), each with three nucleotides in different combinations. There are only 64 ($4 \times 4 \times 4 = 64$) possible codons, of which 61 encode the 20 amino acids and three are stop codons. There are more codons (n = 61) than there are amino acids (n = 20) because some amino acids are encoded by more than one codon (e.g., UCU, UCC, UCA, UCG, AGU, and AGC all encode the amino acid serine). This is known as redundancy in the genetic code.

Essentials of a Gene

Figure 1 shows the key elements of a gene. Exons are numbered from the left (5′, upstream) and are separated by introns. Nucleotides (in groups of three) in exons encode amino acids or "stop," whereas nucleotides in introns are not encoded. Nucleotides in an exon are written in uppercase letters and those in introns and intervening sequences are written in lowercase letters. At the junction of an exon to an intron there is an invariant sequence of four nucleotides (AGgt) called the donor splice site, and at the junction of an intron to an exon is another invariant sequence of four nucleotides (agGT) called the acceptor splice site. The splice sites interact to excise (or outsplice) the introns, thereby converting genomic DNA to mRNA. A single strand of DNA (5′ to 3′) acts as a template and is duplicated exactly to form mRNA. Nucleotide C invariably pairs with G, and A

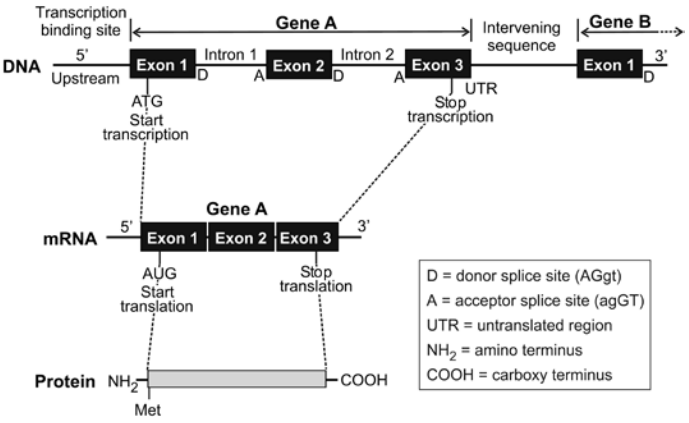

Fig. 1. Anatomy of a gene.

with T. Upstream from the first exon of a gene there are binding sites (promoter regions) for factors that are required for transcription (from DNA to mRNA) of the gene. Transcription of DNA always begins at the ATG, or "start," transcription codon. The promoter region can be ubiquitous, tissue-specific, or switched on under certain circumstances. At the 3′ end of a gene there is a "stop" transcription codon (TAA, TAG, or TGA), and beyond that there is often an untranslated region (UTR). Between adjacent genes on a chromosome there is an "intervening" sequence of nucleotides, which is not transcribed.

After the introns are excised, the resultant mRNA contains nucleotides from the exons of the gene. Nucleotides in mRNA are translated (from mRNA to protein) in sets of three (a codon) to produce a sequence of amino acids, which form a protein. Translation of mRNA always begins at the AUG, or "start," codon and terminates at a "stop" codon (UAA, UAG, or UGA). The resultant protein consists of amino acids starting with methionine (whose codon is AUG) at the amino (NH_2) terminus. Methionine, or a "leader" sequence of amino acids, is sometimes cleaved from the functional protein, and thus a written sequence of amino acids for a mature protein does not necessarily begin with methionine.

Molecular Bases of Blood Groups

Although many mechanisms give rise to a blood group antigen or phenotype (Table 1), the majority of blood group antigens are a consequence of a single nucleotide change. The other mechanisms listed give rise to a small number of antigens and various phenotypes. Figure 2 shows a short hypothetical sequence of DNA together with transcription (mRNA) and translation (protein) products. The effect of a silent (synonymous), missense (nonsynonymous), or nonsense single nucleotide change and examples involving blood group antigens are illustrated.

Table 1. Molecular events that give rise to blood group antigens and phenotypes

Molecular Mechanism	Example for Blood Group
Single nucleotide changes in mRNA	See Figure 2 and Table 2
Single nucleotide change in a transcription site	T>C in GATA of *FY*
Single nucleotide change in a splice site	ag>aa in Jk(a–b–)
Deletion of a nucleotide(s)	See Figure 2 and Table 2
Deletion of an exon(s)	Exon 2 of *GYPC* in Yus phenotype
Deletion of a gene(s)	*RHD* in some D– people
Insertion of a nucleotide(s)	See Figure 2 and Table 2
	37-bp insert in *RHD* (*RHDψ*) in some D– people
Insertion (duplication) of an exon(s)	Exon 3 of *GYPC* in Ls(a+)
Alternative exon	Exon 1 in I– people
Gene crossover, conversion, other recombination events	Many hybrid genes in MNS and Rh systems
Alternative initiation (leaky translation)	GPD
Absence/alteration of a required interacting protein	RhAG in regulator Rh_{null}, and Rh_{mod}
Presence of a modifying gene	*In(Lu)* in dominant Lu(a–b–)
Unknown	K_{null}, Gy(a–)

GPD = Glycophorin D, RhAG = Rh associated glycoprotein, which is required for expression of Rh antigens

Effect of a Single Nucleotide Change on a Blood Group

Owing to redundancy in the genetic code, a silent (synonymous) nucleotide change does not change which amino acid is encoded and, thus, does not affect the antigen expression. Nevertheless, because it is possible that such a change could alter a restriction enzyme recognition site or a primer binding site, it is important to be aware of silent nucleotide changes when designing a PCR-based assay. In contrast, a missense (nonsynonymous) nucleotide change results in a different amino acid, and these alternative forms of the allele encode antithetical antigens. Figure 2 illustrates this for the situation in which G in a lysine codon (AAG) is replaced by C, which gives rise to the codon for asparagine (AAC). The example of a missense nucleotide change shows that a C to T change is the only difference between k and K. A nonsense nucleotide change results in a codon for an amino acid to become a stop codon. Figure 2 and Table 2 give examples relative to blood groups.

Fig. 2. A hypothetical sequence of DNA and the effect of a single nucleotide change.

EFFECT OF DELETION OR INSERTION OF NUCLEOTIDE(S)

A deletion of one nucleotide results in a –1 frameshift and an eventual stop codon (Fig. 2 and Table 2). Typically, this leads to the encoding of a truncated protein, but it can cause elongation. For example, a deletion of C close to the stop codon in the A^2 allele results in a transferase with 21 amino acids more than in the A_1 transferase.[4] Deletion of one nucleotide results in a –1 frameshift and a premature stop codon. Similarly, deletion of two nucleotides results in a –2 frameshift and a premature stop codon. Deletion of a nucleotide can cause a stop codon, but there is no known example for a blood group.

An insertion of one nucleotide results in a +1 frameshift and a premature stop codon (Fig. 2 and Table 2). Insertion of two nucleotides results in a +2 frameshift and a premature stop codon. Insertion of a nucleotide can cause a stop codon, but there is no known example for a blood group.

Table 2. Molecular bases associated with a few blood group antigens

Antigen/ Phenotype	Gene	Nucleotide Change	Amino Acid
Missense nucleotide change			
S/s	*GYPB*	143T>C	Met29Thr
E/e	*RHCE*	676C>G	Pro226Ala
K/k	*KEL*	698T>C	Met193Thr
Fy^a/Fy^b	*FY*	125G>A	Gly42Asp
Jk^a/Jk^b	*JK*	838G>A	Asp280Asn
Do^a/Do^b	*DO*	793A>G	Asn265Asp
Nonsense nucleotide change			
Fy(a–b–)	*FY*	407G>A	Try136Stop
D–	*RHD*	48G>A	Trp16Stop
Gy(a–)	*DO*	442C>T	Gln148Stop
Nucleotide deletion			
D–	*RHD*	711Cdel	Frameshift→245Stop
D–	*RHD*	AGAG	Frameshift→167Stop
Nucleotide insertion			
A_{el}	*ABO*	798-804Gins	Frameshift→Stop
D–	*RHD*	906GGCTins	Frameshift→donor splice site change (I6+2t>a)

Molecular Bases of Blood Group Antigens

The genes encoding 28 of the 29 blood group systems (only P1 remains to be resolved) have been cloned and sequenced.[5] Focused sequencing of DNA isolated from blood obtained from patients or donors with serologically defined antigen profiles has been used to determine the molecular bases of variant forms of the gene. This approach has been extremely powerful because antibody-based definitions of blood groups readily distinguish variants within each blood group system. Details of these analyses are beyond the scope of this paper, but up-to-date details about alleles encoding blood groups can be found on the blood group antigen gene mutation database at: http://www.ncbi.nlm.nih.gov/projects/mhc/xslcgi.fcgi?cmd=bgmut/systems, or by entering "dbRBC" in a search engine. Suffice it to say that there are 29 blood group systems, 34 gene loci, more than 250 antigens, and close to 1,000 alleles that encode the blood group antigens and phenotypes.

Once the molecular basis of a blood group antigen has been determined, the appropriate part of the DNA can be analyzed to predict the presence or absence of a blood group antigen on the surface of an RBC. Fortunately, as the majority of genetically defined blood group antigens are the consequence of a single nucleotide change, simple PCR-based assays can be used to detect a change in a gene encoding a blood group. Figure 3 illustrates examples of read-outs for commonly used assays. Numerous DNA-based assays have been described for this purpose and will be described in a subsequent paper.

Summary

Numerous studies have analyzed blood samples from people with known antigen profiles and

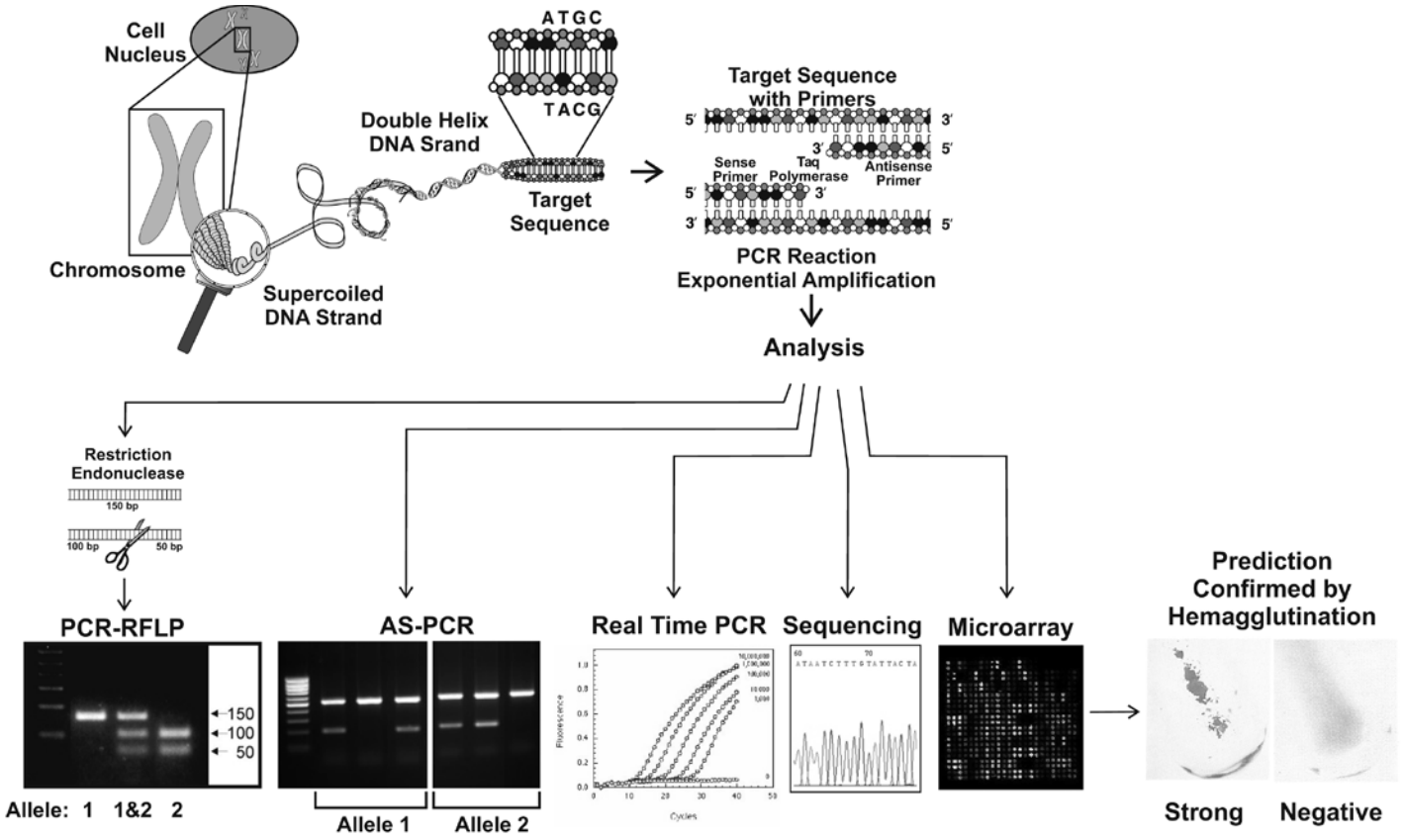

Fig. 3. From cell nucleus to DNA to assays used to predict a blood type.

identified the molecular bases associated with many antigens.[4,6] The available wealth of serologically defined variants has contributed to the rapid rate with which the genetic diversity of blood group genes has been revealed. Although PCR-based assays have limitations, they nevertheless have several clinical applications, which are described in subsequent papers in this issue of *Immunohematology*.

Acknowledgment

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References

1. Hillyer CD, Shaz BH, Winkler AM, Reid M. Integrating molecular technologies for red blood cell typing and compatibility testing into blood centers and transfusion services. Transfus Med Rev 2008;22:117–32.
2. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 1987;155:335–50.
3. Mullis KB. The unusual origin of the polymerase chain reaction. Sci Am 1990;262:56–65.
4. Reid ME, Lomas-Francis C. Blood group antigen factsbook. 2nd ed. San Diego: Academic Press, 2004.
5. Lögdberg L, Reid ME, Lamont RE, Zelinski T. Human blood group genes 2004: chromosomal locations and cloning strategies. Transfus Med Rev 2005;19:45–57.
6. Human Genome Variation Society. NCBI, dbRBC. Available at: http://www.ncbi.nlm.nih.gov/projects/mhc/xslcgi.fcgi?cmd=bgmut/home. Accessed November 1, 2008.

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Principles of PCR-based assays

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DNA-based assays are powerful tools to predict the blood group of an individual and are rapidly gaining in popularity. DNA, which can be extracted from various sources using commercial kits, is amplified by PCR to obtain a sufficient amount of the target of interest for analysis. There are different types of PCR assays: standard single PCR (followed by RFLP or sequencing), allele-specific PCR, multiplex PCR, and real-time PCR. Microarray platforms are a newer application of molecular testing, popular because they analyze multiple nucleotides in a single assay and have a high-throughput potential. This review briefly describes the principles of PCR-based assays that are commonly used in transfusion medicine. ***Immunohematology* 2008;24:170–175.**

Key Words: DNA, PCR, BeadChip, RFLP, blood group sequencing, molecular analysis

The molecular genetics world was revolutionized in 1983 with the advent of PCR,[1] which allows the amplification of DNA and analysis of genes. Since the development of this tool, the molecular genetics field has greatly expanded, and the method has been used to answer a variety of questions in numerous areas, including forensics, evolution, archeology, and, in recent years, transfusion medicine.

RBC agglutination is still the conventional test method for detecting blood group antigens and antibodies as it is relatively inexpensive and quick and requires minimal equipment. However, it is not without its limitations. Typing multiply transfused patients, a lack of rare or reliable potent antisera, and encountering reagent discrepancies are just some situations in which serology is not straightforward. Molecular testing is useful in these instances, and it can be applied now that the genes encoding many of the blood group antigens have been cloned and sequenced. Also, most blood group antigens are differentiated by single-nucleotide polymorphisms (SNPs) at the DNA level that change the amino acid at the protein level, and these polymorphisms can be targeted using PCR assays. The applications are further described in other papers in this series.[2–4]

Test Environment and Equipment

Unlike traditional blood bank laboratories where most work can be done at a single workstation, molecular laboratories must separate "clean" and "dirty" processes, and, ideally, locate each in a separate room. "Clean," or pre-PCR, processes include DNA extraction and PCR setup. "Dirty," or post-PCR, processes include analysis or any testing done on the PCR-amplified products. Separation is necessary to prevent contamination among samples and, most importantly, to prevent PCR-amplified products from contaminating patient and donor DNA samples. The different processes must have separate equipment and supplies to further minimize contamination. Laboratories use micropipette filter tips to minimize aerosols and decontaminate surfaces and equipment with dilute bleach or a DNA removal agent. UV light exposure can also be used on surfaces and equipment to degrade any potential contaminating DNA.

Extraction of DNA

Genomic DNA can be extracted from various sample sources such as peripheral whole blood, urine, amniotic fluid, buccal swabs, and dried blood spots on filter paper (QIAamp DNA Blood Mini Kit, QIAGEN, Valencia, CA). For blood samples, EDTA is the preferred coagulant, but other coagulants, such as citrate, are acceptable. Lithium heparin is discouraged because heparin can interfere with the PCR reaction. DNA is very stable, and samples sent for molecular testing can be weeks or months old, but optimal samples are less than 3 days old, stored at refrigerator temperatures.

There are many commercial kits available that make the process of extracting genomic DNA relatively easy. Compared with early methods that used desalting and phenol/chloroform, the extraction process is now fast, reliable, and nontoxic. DNA extraction using commercial kits removes proteins and other contaminants, which may inhibit PCR, and yields DNA of high quality in less than 1 hour.

Polymerase Chain Reaction (PCR)

PCR is used to amplify a specific region of DNA that harbors the nucleotide(s) of interest, i.e., for a blood bank, this is the region that encodes the blood group antigen. The PCR reaction mixture consists of a reaction buffer, magnesium chloride, deoxynucleotide-triphosphates (dNTPs) (G, A, T, C), oligonucleotide primers complementary to the gene of interest, heat-stable enzyme *Taq* polymerase (which is isolated from the bacterium *Thermus aquaticus*), and the DNA of interest. Primers typically range from 18 to 36 base

pairs (bp) and are designed to amplify relatively short DNA segments, usually 200 to 800 bp in length. The tube with the PCR reactants is placed in a thermal cycler programmed for a series of cycles that consist of heating at 95°C to denature the DNA, followed by cooling at 55° to 65°C to anneal the primers to the DNA template, and heating again to 72°C for the heat-stable *Taq* polymerase to synthesize the DNA between the primers. Figure 1 outlines the principles of PCR amplification. In step 1, the denaturation step, the double-stranded DNA is made single-stranded by the high temperature of 95°C. In step 2, the annealing step, the oligonucleotide primers, which are complementary to the gene target of interest, bind to the single-stranded DNA. The annealing temperature used is dependent on the length, nucleotide sequence, and particularly the G/C content, of the primers and typically ranges from 55°C to 65°C. In step 3, the extension step, the *Taq* polymerase adds dNTPs to the end of the primer complementary to the DNA template to which it annealed, thereby creating an exact copy and double-stranded DNA. These steps are repeated 25 to 35 times, which results in an exponential increase in the number of copies of the specific area of interest (PCR amplicon). The PCR products are subjected to analysis by any number of different methods, which can include electrophoresis to separate the fragments through an agarose gel, based on size, with the fragment bands visualized by ethidium bromide staining and UV light. Alternatively, the products may be digested with enzymes, sequenced directly, or read by automated readout systems (discussed in a later section).

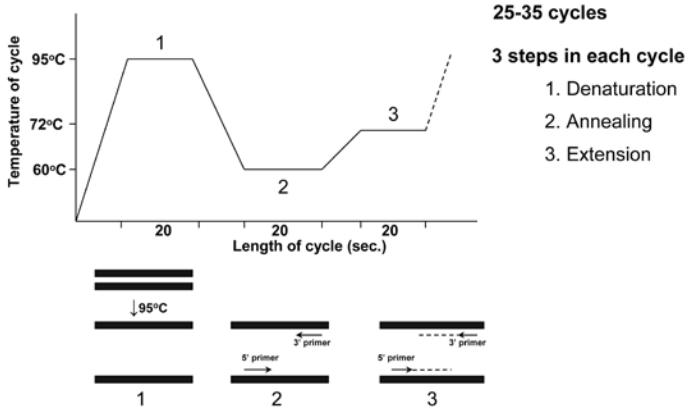

Fig. 1. Principle of PCR amplification. (1) Denaturation step at 95°C to separate double-stranded DNA. (2) Annealing step typically at 55° to 65°C for binding of primer to single-stranded DNA. (3) Extension step at 72°C for the creation of an exact double-stranded DNA copy.

Manual PCR Assays

Various PCR assays commonly used include allele-specific (AS)-PCR, PCR-restriction fragment length polymorphism (RFLP), and multiplex PCR. AS-PCR requires two reactions to be set up for each DNA sample. In this type of PCR assay, each reaction tube has one primer that is gene-specific (i.e., common to both alleles), and one primer that is specific for one of the two possible alleles present. After PCR amplification, no additional manipulation of the PCR products is required, and they are visualized by agarose gel electrophoresis (Fig. 2). Because the lack of a PCR product indicates an allele is not present, a positive internal control consisting of primers that will amplify a product in all samples is needed as a control for the PCR reaction.

In PCR-RFLP methods, one PCR reaction is performed and the two primers are gene-specific. The alleles are differentiated after PCR by digestion of the products with a restriction enzyme. Restriction enzymes, which are commercially available, are highly specific and cleave a unique 4- or 6-bp sequence of nucleotides (Fig. 3A). Alleles are discriminated by the fragment pattern visualized by gel electrophoresis after digestion. The fragment pattern is compared with those of known controls tested in parallel. The controls used are generally a homozygous allele A, a homozygous allele B, and a heterozygote A/B (Fig. 3B).

Typically, AS-PCR and PCR-RFLP target a single allele. Multiplex PCR enables simultaneous amplification of many target alleles or regions of DNA in one reaction by using multiple primer pairs. This allows for a reduction in the number of different assays performed and saves time and reagents. However, multiplexing has limitations in the number of primer pairs that can be combined in one reaction, and the initial optimization of multiplex assays can be technically challenging and difficult. Primer annealing temperatures need to be similar, and all the reaction components must be carefully optimized to avoid false-priming of primers and amplified products. After multiplex PCR, products are visualized on agarose gels and discriminated by fragment sizes.

Sequencing

Another often-used PCR analysis involves purification followed by direct gene sequencing of the PCR product. Sequencing is an automated procedure, typically performed by a sequencing facility. It

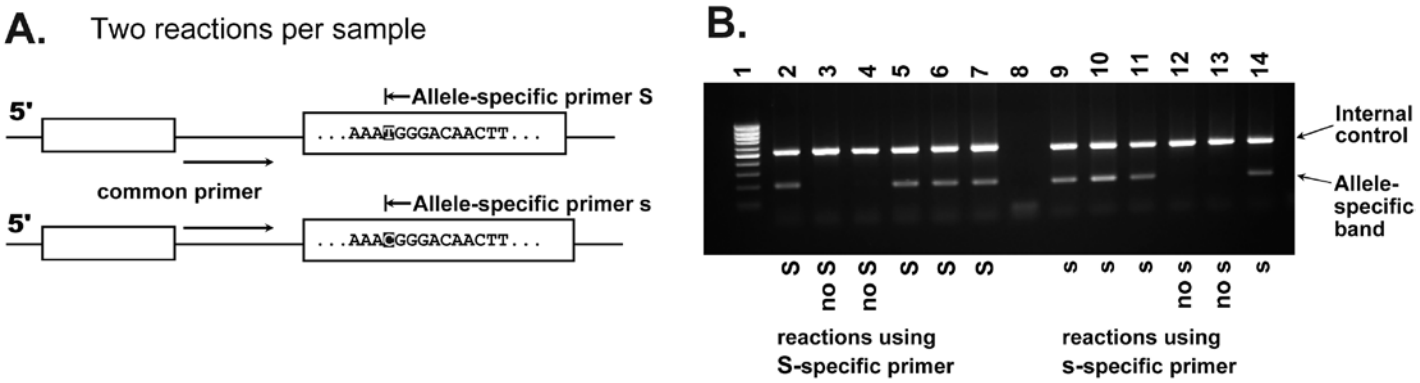

Fig. 2. Allele-specific PCR assay. **A.** Two reactions are set for each sample. Each reaction has a gene-specific primer common to both reactions and an allele-specific primer for the allele of interest. **B.** AS-PCR reactions run on a 1% agarose gel. (Lane 1) DNA ladder; (lanes 2–7) PCR products from six samples using a *GYP*B*^{S} allele-specific primer; (lane 8) negative control containing no DNA; (lanes 9–14) PCR products from same six samples using *GYP*B*^{s} allele-specific primer; and negative control containing no DNA. An additional pair of primers is used as an internal control for PCR amplification and the product is not related to the gene of interest.

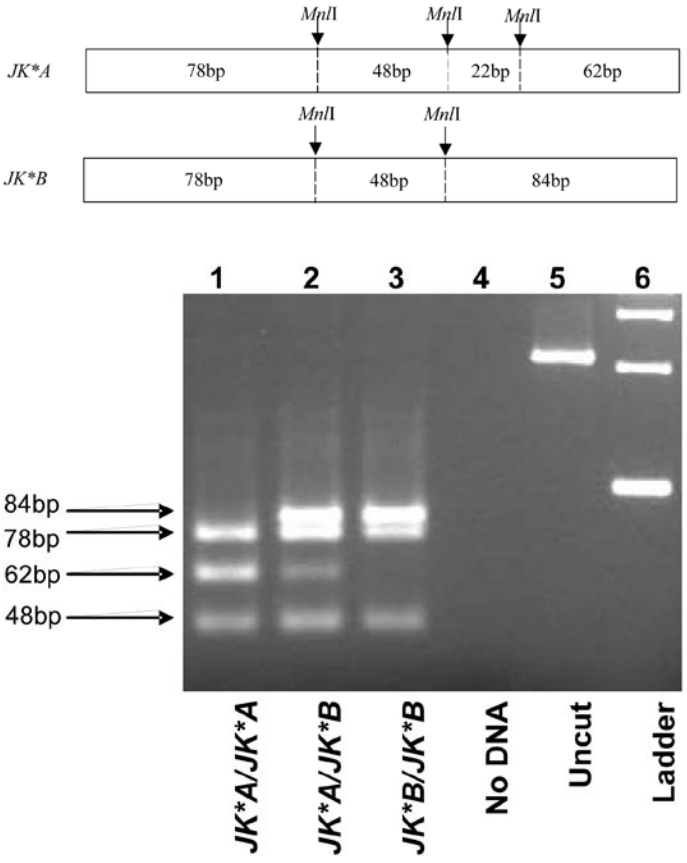

Fig. 3. PCR-RFLP. **A.** Recognition sites for allele *JK*A* and allele *JK*B* are shown. Digestion with the restriction enzyme, *Mnl*I, will discriminate between the two alleles. *JK*A* will be cleaved in three positions, resulting in four fragments, whereas *JK*B* will be cleaved in two positions, resulting in three fragments. **B.** Reactions run on a 2% agarose gel. The ability to visualize the 22-bp fragment depends on the type and concentration of the gel. (Lane 6) DNA ladder; (lane 5) DNA amplicon not digested with the restriction enzyme; lane 4) no DNA control; (lanes 1–4) PCR products digested with the restriction enzyme, *Mnl*I.

involves an additional PCR performed on the initial PCR products using dideoxynucleotides (ddNTPs) that are fluorochrome-labeled bases. Each base (A, C, T, and G) is represented by a color peak on a scan, which is known as an electropherogram scan (Fig. 4). In this method, the nucleotide sequence of the PCR product is interpreted by comparison to the known sequence. It is optimal for investigating multiple changes in a specific region of DNA and identifying previously unknown nucleotide changes in the gene sequence.

Real-Time PCR

In real-time PCR, the amplified products are monitored in real time by measuring the fluorescence emitted by a reporter molecule after each cycle of amplification. There are different types of fluorescent reporter molecules, including those that bind to double-stranded DNA molecules (i.e., SYBR Green) and those that bind to specific DNA sequences (i.e., Molecular Beacons or TaqMan Probes), which can be purchased from various commercial vendors. Unlike PCR assays already described, real-time PCR is a semiautomated method that does not require an additional gel electrophoresis step; rather, the fluorescence intensities are monitored and interpreted by computer.

SYBR Green

Real-time PCR reactions contain reagents similar to those in the PCR assays described earlier but with the addition of a fluorescent dye. SYBR Green has little fluorescence, but when the dye is bound to double-stranded DNA, fluorescence is emitted. After

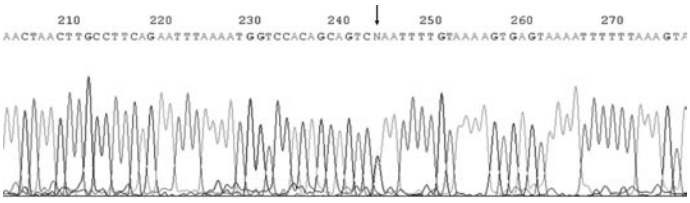

Fig. 4. Electropherogram of a sequence of DNA. Each base (A, C, T, and G) is represented by a color peak on a scan, and the nucleotide sequence is displayed. In a single position with two peaks, an "N" (indicated by arrow) shows that the sample is heterozygous for nucleotides A and G at that position.

each PCR cycle, the fluorescence emitted is proportional to the PCR product amount. SYBR Green is inexpensive and binds to all double-stranded DNA. Nonspecific PCR products can be differentiated from PCR products by melting-curve analysis. Multiplexing is not possible.

TaqMan Probes

Real-time PCR reactions involve using DNA-specific probes that can be labeled with different color dyes (TAM, TET, JOE, VIC, SYBR Green), which allows for multiplexing. Sequence-specific probes, such as TaqMan probes, are labeled with a reporter fluorescent dye and a quencher dye. When the two dyes are in close proximity, there is little fluorescence. If an allele is present and the probe binds during the PCR reaction, the *Taq* polymerase cleaves the reporter dye, separating it from the quencher and emitting fluorescence (Fig. 5). Although TaqMan probes allow for multiplexing, the number of targets is limited by the number of fluorescent dyes commercially available (typically two to four) and by the number of colors recognized by the instrument. Characteristics of the different PCR assays are summarized in Table 1.

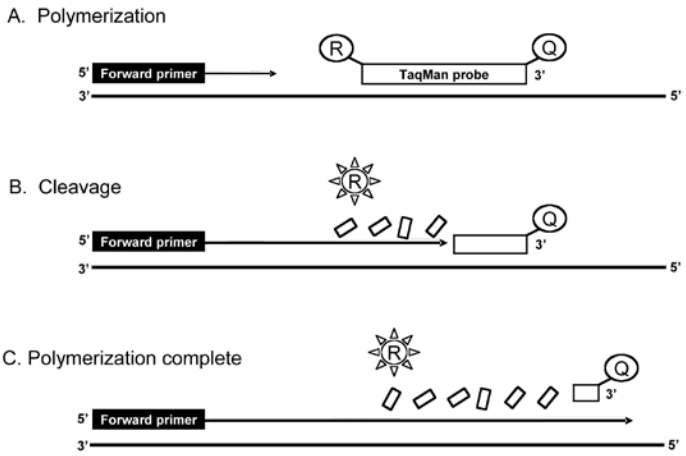

Fig. 5. Real-time PCR with sequence-specific probes. **A.** Sequence-specific probe with reporter (R) and quencher (Q) dye bound to the DNA strand. Little fluorescence is emitted. **B** and **C.** PCR product extends and the *Taq* polymerase cleaves the reporter dye and separates it from the quencher dye. Fluorescence is emitted.

Automation

Manual PCR methods, such as AS-PCR and PCR-RFLP, are labor- and time-intensive, and PCR sequencing and real-time PCR are costly. Determination of numerous polymorphisms covering multiple systems with these assays would take days, making the possibility of mass donor screening impossible. Therefore, automation of the process is needed for rapid, cost-effective typing.

DNA extraction is also a labor- and time-intensive process. Several commercial companies have automated robotic instruments for extraction of 96 samples in as little as 2 hours. These instruments have similar chemistries to those of the manual extraction kits.

Microarray and BeadChip platforms have recently been introduced for the automation of prediction of

Table 1. Characteristics of various PCR assays

Characteristic	AS-PCR	PCR-RFLP	Multiplex	Sequencing	Real-Time PCR	Array Platforms
Manual process	√	√	√			
Labor- and time-intensive	√	√	√	√		
Extensive optimization	√		√		√	
Quantification					√	
Single SNP	√	√	√			
Multiple SNPs			√	√	√	
High throughput						√
Requires additional equipment				√		√
Donor screening						√

AS-PCR = allele-specific polymerase chain reaction; PCR-RFLP = polymerase chain reaction–restriction fragment length polymorphism; SNP = single-nucleotide polymorphism.

blood group antigens. The steps in testing include an initial multiplex PCR reaction, followed by hybridization to oligonucleotide probes either bound to glass slides or bound to beads, with fluorescence signals indicating the presence or absence of an allele.

The platform from Progenika (BloodGen Project; Cambridge, MA) includes allele-specific oligonucleotide probes bound to a modified glass slide. The PCR involves several separate multiplex reactions that simultaneously amplify numerous targets for nine blood group systems. The blood group–specific primers are labeled at the 5′ region with a universal tag that allows for increased specificity and more consistent yield of all PCR products. After amplification, the PCR products are labeled with Cy fluorescent dyes, fragmented with DNAse I, hybridized to the glass slides, and washed. An array scanner scans the slides, and the software reads the fluorescent intensity to identify alleles and determine genotypes and predicted phenotypes. Currently, the Progenika BLOODchip targets 116 SNPs for ABO, RHD (common weak D, partial D, Del, and D negative), RHCE (C/c, E/e, VS, V, CX, CW), Kell (K/k, $Js^{a/b}$, $Kp^{a/b/c}$, Kmod-1), Kidd, Duffy ($Fy^{a/b/x}$, Fy null), MNS (M/N, S/s, U, Gp.Mur), Dombrock ($Do^{a/b}$), Colton ($Co^{a/b}$), and Diego ($Di^{a/b}$).[5]

BioArray Solutions Ltd. (Warren, NJ), now part of Immucor, developed a BeadChip platform that uses color-coded beads coated with allele-specific oligonucleotides. These beads are randomly immobilized onto a silicone wafer chip (BeadChip). One multiplex PCR reaction is used to amplify the blood group targets. After the PCR, the products are made single-stranded and hybridized to the BeadChip. If the PCR products are complementary to the oligonucleotide, indicating the allele is present, a DNA polymerase will extend the strand and incorporate fluorescently labeled dNTP. A snapshot of fluorescent intensities is taken and decoded using the software that determines the intensity ratio for paired probes, interprets the data, and interprets the results as both a genotype and predicted phenotype. The process from start to finish is approximately 5 hours.[6] Both automated platforms are substantially faster than manual PCR assays and allow for high-throughput testing. The Human Erythrocyte Antigen (HEA) BeadChip includes probes that detect the polymorphisms for K/k, $Kp^{a/b}$, $Js^{a/b}$, $Jk^{a/b}$, Fy^{a}/Fy^{b}/weakened Fy^{b}/Fy GATA, M/N, S/s/UVAR, Rh C/c and E/e, VS and V. These platforms cannot detect rare alleles or silenced alleles unless the polymorphisms responsible are specifically targeted. For example, the current HEA BeadChip targets only common silencing alleles in *FY*B* (GATA –33 t>c) and *GYP*B* (S–s–UVAR intron 5 +5g>t and 230C>T).

Conclusion

Agglutination will not become obsolete in blood banking as molecular testing is a prediction of the RBC phenotype and cannot replace antibody identification and screening. Molecular analysis is a valuable adjunct to hemagglutination for the prediction of antigen status or resolution of a discrepancy,[7] and it is an important tool for resolving complex serology cases, typing multiply transfused patients, and performing large-scale donor screening.

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References

1. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 1987;155:335–50.
2. Lomas-Francis C, DePalma H. 2007 Rock Øyen Symposium: DNA-based assays for patient testing: their application, interpretation, and correlation of results. Immunohematology 2008; 24:180–90.
3. Straus D, Reid ME. 2007 Rock Øyen Symposium: Values of DNA-based assays for donor screening and regulatory issues. Immunohematology 2008;24:175-9.
4. Westhoff CM. 2007 Rock Øyen Symposium: The potential of blood group genotyping for transfusion medicine practice. Immunohematology 2008;24:190-5.
5. Avent ND, Martinez A, Flegel WA, et al. The BloodGen project: toward mass-scale comprehensive genotyping of blood donors in the European Union and beyond. Transfusion 2007;47:40–6S.
6. Hashmi G, Shariff T, Seul M, et al. A flexible array format for large-scale, rapid blood group DNA typing. Transfusion 2005;45:680–8.
7. Westhoff CM. Molecular testing for transfusion medicine. Curr Opin Hematol 2006;13:471–5.

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Value of DNA-based assays for donor screening and regulatory issues

D. Strauss and M.E. Reid

Hemagglutination, the gold standard method to detect the presence or absence of blood group antigens on RBCs, has served the transfusion community well for decades. It is simple, and, when done correctly, it has a specificity and sensitivity that is appropriate for most testing in the vast majority of patients requiring blood transfusion. The limitations of hemagglutination for screening donor blood include that both testing and data entry are labor-intensive, that the required antibody is not always commercially available, and that it may be limited in volume, weakly reactive, or costly. These scenarios can make it difficult to screen for large numbers of antigen-negative blood donors. The knowledge of the molecular bases of blood group antigens makes it possible to screen donors to predict their antigen status. High-throughput platforms provide a means to test relatively large numbers of donors, thereby opening the door to change the way antigen-negative blood is provided to patients. This review discusses testing for blood group antigens by hemagglutination and bead chip technology. It also reviews regulatory issues, including validation and training, and suggests an algorithm for screening and confirming blood types of donors. ***Immunohematology* 2008;24:175–179.**

Key words: DNA testing, donor testing, blood group antigens, hemagglutination

Typing for Blood Group Antigens in Donors

Testing for the presence or absence of a blood group antigen has traditionally been done by phenotyping using serologic methods. However, it is now possible to predict the presence or absence of a blood group by assaying DNA. There are two basic types of PCR-based assays: laboratory-developed tests (LDTs), which were previously known as "homebrew" assays, and DNA arrays.

The decision as whether to use hemagglutination or DNA methodologies is dependent on various things, such as availability of reagents and the complexity of the molecular basis of an antigen's phenotype. For example, because of the complexity of genes encoding antigens and phenotypes in ABO and Rh blood group systems, DNA analysis is not the method of choice for routine ABO and D determination. However, screening for donors by analyses of DNA is valuable for typing of the so-called minor blood group antigens and Rh variants, and for resolving apparent discrepancies.

Use of DNA-Based Assays to Predict the Antigen Type in Donors

DNA-based assays, with their high-throughput capability, can be used to mass screen donors, and they provide a tool to enable us not only to increase the antigen-negative inventory of combinations of the minor antigens but also to find donors whose RBCs lack a high-prevalence antigen, namely, U, U^{Var}, Fy3, Lu^b, Di^b, Hy, Jo^a, Sc1, LW^a, or Co^b. This can lead to improved patient care by having the appropriate antigen-negative blood available.[1] By using these high-throughput DNA-based methods as a screening tool, precious antisera can be conserved to be used to serologically confirm the results predicted by DNA typing.

DNA-based assays also can be useful to detect genes that are predicted to encode weakly expressed antigens and thereby prevent immunization or possible transfusion reactions. As we now have options for how to test for antigen-negative donors, it is necessary to determine which method to use under which circumstances. Table 1 lists suggestions for when to use hemagglutination and when to use molecular methods.

Testing for minor antigens

When the molecular basis of a blood group antigen has been determined to be caused by a single nucleotide change or small deletion or insertion of nucleotides, simple PCR-based assays can be used to predict their presence or absence on RBCs. There are numerous examples of this, e.g., K/k, Fy^a/Fy^b, Jk^a/Jk^b, S/s, Do^a/Do^b, and Yt^a/Yt^b.[2] PCR-based assays are particularly valuable to predict antigens when the available antibody is weak or the antibody is not readily available, e.g., anti-Do^a, -Do^b, -Js^a, -Js^b; -C^w, -V, and -VS. These assays are particularly valuable in the

Table 1. Antigen typing by hemagglutination or molecular assays

Hemagglutination	Molecular assays
Readily available antisera, e.g., Anti-k, -Kp^b, -Js^b, and -Fy3	Antisera not readily available, e.g., anti-Lu^b, -Di^b, -Do^a, -Do^b, -Hy, -Jo^a, -Co^a, -Sc1, -Yt^a ,Cw ,V, and VS
Molecular basis unknown, e.g., Vel–, Lan–, At(a–), or Jr(a–)	Need high throughput, e.g., for any blood group where the molecular basis is relatively simple
Molecular bases known but multiple backgrounds, e.g., null phenotypes such as Rh_{null}, K_0, Gy(a–), or McLeod	

Dombrock blood group system; typing for Do^a, Do^b, Hy, and Jo^a by hemagglutination is notoriously difficult because the corresponding antibodies, although clinically significant, are often weakly reactive, available only in small volume, and in sera containing other alloantibodies. Testing for Do antigens by DNA analyses is accomplished without the need for special reagents (antibodies) and is the first example of where PCR-based assays surpass hemagglutination for antigen typing.[3]

To meet the antigen-negative RBC component needs of chronically transfused, immunized African American patients, it has been the practice to use RBC components from donors of the same ethnic origin. For such patients, a commonly needed phenotype is C–, E–, S–, K–, Fy(a–), and Jk(b–), which is most likely to be found in people of African origin. However, these donors have immunogenic antigens on their RBCs that are of low prevalence in the random population but present in up to 20 percent in African Americans.[2] Thus, we find that many of these patients now have made anti-Js^a, -V/VS, -Go^a, and -DAK, and thus require RBC components lacking these antigens. Providing the RBC components lacking these antigens is difficult for several reasons: (1) patients make antibodies to these antigens in addition to several others, e.g., anti-C, -K, -Fy^a, and -Jk^b; (2) the antigens are not on antibody screening RBCs; and (3) the crossmatch is not always reliable in their detection. Thus, although African American donors are the best place to look for certain combinations of antigen negativity for transfusion to African Americans, their RBCs are likely to express these low-prevalence antigens.

Some of the numerous variants in the Rh blood group system are clinically relevant but difficult to distinguish by hemagglutination. Of particular note are hr^S and hr^B, the absence of which are known to be encoded by several distinct alleles.[4] Although several assays are required, DNA-based assays can be used to differentiate these alleles. This has the potential to be able to provide donor blood that is precisely matched to that of a patient.

Resolution of ABO and Rh discrepancies

DNA-based assays can be used to detect alleles that encode a blood type that is detected by some antibody reagents but not others. Resolution of these apparent discrepancies is helpful to reveal that the different results obtained by hemagglutination are caused by a genetic variant (not reportable to the Food and Drug Administration [FDA]) and not by a reagent failure or technologist error (reportable to the FDA). This is particularly pertinent to A, B, D, C, and e.

DNA Array Platforms

DNA arrays make it possible for multiple assays to be performed on one sample simultaneously so a large number of samples can be tested for a large number of nucleotide changes. There is a low incremental cost for each assay that is added to a DNA array. Another advantage of this technology is that results can be analyzed and interpreted by computer, and there is the potential to directly download interpretations to a donor database.

In 2007, 2355 donors of known genotypes covering 24 RBC antigens in 10 blood group systems were tested using a DNA array (HEA BeadChip, BioArray Solutions, Warren, NJ).[5] There was concordance of 4510 of 4534 antigen typings (99.5%). The discordances were attributable mostly to clerical errors found on the original reports, and for Ss as a result of silencing mutations, which is included in the HEA 1.1 BeadChip. This was a joint venture with the New York Blood Center (NYBC) and BioArray Solutions.

Validation of BioArray Solutions HEA 1.1 BeadChip at NYBC

The HEA 1.1 BeadChip was initially validated by NYBC, extracting DNA from 396 donor samples, with BioArray Solutions performing the assay. Validation results are shown in Table 2. Of the 8578 successful tests, 62 percent were predicted to be antigen-positive and 38 percent were predicted to be antigen-negative. Sixty-seven percent of the negative results were new results (no previous serology testing). Of particular note, the testing revealed six new Sc1– donors, as well as three e– and many c–, and s– donors that have been confirmed by serology.

Table 2. NYBC validation results for HEA 1.1 BeadChip

Test name	Successful tests	Positive results	Negative results	New negative results	Agreement	Conflicts*
Antigen test C	104	104	0	0	0	0
Antigen test c	369	266	103	30	73	1
Antigen test Co^a	377	377	0	0	0	0
Antigen test Co^b	377	16	361	359	2	0
Antigen test Di^a	358	10	348	348	0	0
Antigen test Di^b	358	350	8	8	0	0
Antigen test Do^a	373	189	184	183	1	0
Antigen test Do^b	373	189	184	184	0	1
Antigen test E	355	54	301	73	228	1
Antigen test e	355	343	12	3	9	0
Antigen test Fy^a	371	169	202	48	154	1
Antigen test Fy^b	370	193	177	50	127	1
Antigen test Hy	373	373	0	0	0	0
Antigen test Jk^a	375	298	77	31	46	1
Antigen test Jk^b	373	229	144	46	98	0
Antigen test Jo^a	373	373	0	0	0	0
Antigen test K	379	15	364	96	268	0
Antigen test k	379	378	1	0	1	0
Antigen test Lu^a	299	10	289	289	0	0
Antigen test Lu^b	301	301	0	0	0	0
Antigen test LW^a	365	365	0	0	0	0
Antigen test LW^b	363	0	363	363	0	0
Antigen test M	366	265	101	94	7	0
Antigen test N	373	241	132	132	0	0
Antigen test S	371	133	238	66	172	2
Antigen test s	371	340	31	19	12	0
Antigen test Sc1	377	371	6	6	0	0
Totals	9578	5952	3626	2428	1198	8

*Conflicts were not resolved since HEA BeadChip 1.2 became available.
Note: Agreement column indicates the number of samples that had serologic results performed and were concordant with molecular results.

A second validation of the HEA 1.1 BeadChip was subsequently performed with NYBC personnel. The operational qualification (OQ) consisted of 68 samples with known RBC phenotypes, which consisted of C/c, E/e, M/N, S/s, K/k, Fy^a/Fy^b, and Jk^a/Jk^b. Included in the OQ were six known samples (cell line) and controls tested in replicate. The cell lines were provided by BioArray Solutions. For the run to be considered valid, all cell line sample results were required to match the panel key and the negative control was required to be negative. Sixty-seven of the 68 samples were found to be concordant with the historic records. One sample did not correlate with the HEA 1.1 BeadChip test result. Historically the sample tested c–, E– using serologic testing. The molecular test predicted that the sample would possess c and E antigens. Repeat hemagglutination and DNA testing by LDT confirmed both results. This sample is being investigated further, but the lack of correlation is likely attributable to a silenced *RHCE*cE* allele. For performance qualification, NYBC tested 960 specimens with unknown phenotypes. Once tested, the results were confirmed with serology. There were no discordant results.

Regulatory Issues

The regulatory items that must be considered include state or city department of health regulations, as well as the FDA Code of Federal Regulations (CFR). The new technology enables the user to obtain many more results than previously possible, therefore requiring decisions to be made regarding how to use the test results. An example would be when a Do(a–) or Do(b–) donor is identified using the BioArray technology. A decision needs to be made regarding the use of the component, the donor's subsequent donations, and how to manage the unlicensed results in the computer system. Suggestions regarding these decisions are discussed below.

New York State Department of Health (NYS-DOH)

The submission to NYS-DOH for test approval consists of providing documentation regarding methods used (standard operating procedures, equipment, reagents, assay limitations), sample requisition forms, result reports with disclaimer statements, references, validation protocol, results, and quality assurance measures. Depending on the state regulations, it may also require the applicant to specify whether the testing will be performed on patient or donor samples. Unless the testing is being used to determine disease status, the analyte applied for under the laboratory license is immunohematology, not molecular testing. This approval allows NYBC to use the tests for clinical purposes. No separate consent is required from the donor. This applies to New York State; local laws may differ.

Table 3. FDA classification of procedures

Licensed reagents	Investigation use only (IUO)	Research use only (RUO)
Hemagglutination	Hemagglutination LDT* DNA tests	Hemagglutination LDT DNA tests Microarrays
Approved for clinical use	Restricted clinical use Not the sole test for clinical use	Not the sole test for clinical use

*LDT = laboratory-developed test.

Blood group typing: FDA compliance

The FDA classifies test procedures into three categories that define when a test may be used for clinical use. These are summarized in Table 3. Hemagglutination is listed in each category because it depends on the source of the reagent. FDA-approved reagents are in the first column and do not need to be confirmed by another method, those prepared in-house (as used in most reference laboratory testing) are in the second column, and those that have not been characterized and validated belong in the third column. Tests performed using investigation use only (IUO) or research use only (RUO) reagents should be confirmed with a licensed reagent if available. If such a reagent is not available, the crossmatch will suffice.

Testing algorithm used at NYBC

The algorithm below is an approach used at the NYBC that allows efficient and effective use of reagents and technology. The testing used depends on the availability of reagents.

1. First test by hemagglutination using a licensed reagent; confirm by hemagglutination with a second licensed reagent on a second donation.
2. First test by hemagglutination using an unlicensed reagent because no licensed reagent is available; confirm by hemagglutination with a second unlicensed reagent (or with the same one, if that is all that is available) or confirm with unlicensed BeadChip, both on a second donation.
3. First test by BeadChip unlicensed when there is limited or no reagent available; confirm with licensed reagent on same donation if possible. Test subsequent donation with BeadChip assay.

Possible handling of results

Table 4 describes an algorithm also used at the NYBC for when the component as antigen-negative and addresses when to link the results to the donor's record. This table takes into consideration the algorithm above for effective use of reagents and will only link results to the donor when two different donations have been tested with the same results. The table demonstrates that a donation may be labeled with an antigen-negative attribute as long as that donation has been tested with a licensed reagent. Once a second donation from the same donor is tested and confirmed to be antigen-negative, additional testing does not have to be performed to attach this attribute to future donations.

Table 4. Algorithm for donor testing and component labeling

Test	No. of times tested for Ag	Donation tested	Label component	Linked to donor
Licensed available	1st	1	Yes	No
Confirm with licensed	2nd	2	Yes	Yes
Unlicensed/license not available	1st	1	Yes	No
Unlicensed/license not available	2nd	2	Yes	Yes
Unlicensed available	1st	1	No	No
Confirm with licensed	2nd	1	Yes	No
Confirm with licensed or unlicensed	3rd	2	Yes	Yes

Other Considerations

Training and competency

As with all new technologies a training and competency program must be adhered to. The vendor should be included in this endeavor. It is the responsibility of the director of the laboratory, and not of the vendor, to ensure results are accurate.

BioArray Solutions offers a 4-day training program. Trainees are given a panel with known results to test, and they receive a certificate of competency on completion. As with any laboratory testing, staff competency testing must be repeated in 6 months and then annually.

Maintenance of test results

The automation of molecular testing generates an abundant amount of information. Great consideration must go into how to manage the results. NYBC has developed a computer program that searches for results by chip name, carrier ID, or date. Any combination of antigen results may be chosen to find components or donors of interest. The program is able to identify new negative results and provide

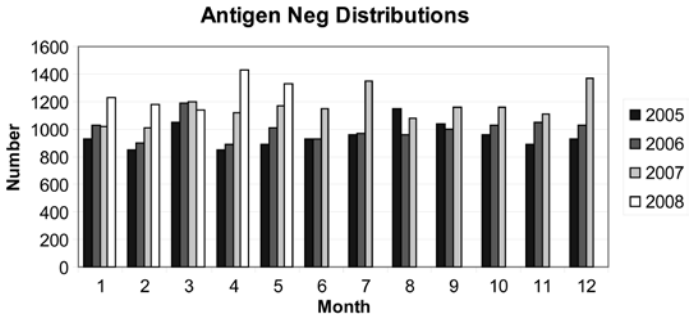

Fig. 1. Histogram showing the number of antigen-negative RBC components distributed by month per year through May 2008.

the location of retention tubes for ease of retrieval; these tubes are then tested serologically to confirm the molecular prediction. In addition, it gives the user the capability to review and override results to ensure proper use of the information. NYBC has additional software that assists the user in avoiding the release of incompatible blood.

If a patient is found to have a clinically significant antibody, a test result may be entered into the blood bank system for that patient. As a consequence of the test result, the software prohibits the distribution of an incompatible blood component to the patient.

If a blood component is requested for a patient that has not been tested by the blood center, the order entry may include a specific phenotype. If units are selected that do not match the requested phenotype, the order cannot be filled and prompts the user.

Patient Care and Component Availability

To maintain an adequate inventory of antigen-negative RBC components, NYBC currently screens the following donor samples per week. The program previously described provides us with a means by which to select samples from donors who have not been previously tested for these antigens. By hemagglutination, we screen approximately 600 group O and group A donor samples in total for 11 antigens (C, c, E, e, K, Fy^a, Fy^b, Jk^a, Jk^b, S, s) for "common" combinations and more than 200 donor samples for 15 high-prevalence antigens (U, hr^B, hr^S, Rh17, k, Kp^b, Js^b, Yt^a, Gy^a, Hy, Jo^a, Ge, PP_1P^k, Vel, Lan). By PCR-based assays, we screen approximately 10 samples by LDT for V, VS, hr^B, hr^S, Do^a, Do^b, Hy, and Jo^a, and approximately 90 by HEA BeadChip per week.

With this amount of screening, we can fill the majority of requests for antigen-negative RBC components. See Figure 1 for distribution of antigen-negative RBC components. However, there are still occasions when an order can only be partially filled and discussion with the patient's physician is necessary. New ways of testing patients and donors with the strategies discussed in this paper should allow for increased component availability and subsequent improved patient care.

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References

1. Lomas-Francis C, DePalma H. 2007 Rock Øyen Symposium: DNA-based assays for patient testing: their application, interpretation, and correlation of results. Immunohematology 2008; 24:180–90.
2. Reid ME, Lomas-Francis C. Blood group antigen factsbook. 2nd ed. San Diego: Academic Press, 2004.
3. Reid ME. Complexities of the Dombrock blood group system revealed. Transfusion 2005;45 (Suppl):92–9S.
4. Westhoff CM. The structure and function of the Rh antigen complex. Semin Hematol 2007; 44:42–50.
5. Hashmi G, Shariff T, Zhang Y, et al. Determination of 24 minor red blood cell antigens for more than 2000 blood donors by high-throughput DNA analysis. Transfusion 2007;47:736–47.

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DNA-based assays for patient testing: their application, interpretation, and correlation of results

C. Lomas-Francis and H. DePalma

DNA analysis for the prediction of RBC phenotype has broad implication in transfusion medicine. Hemagglutination testing, long the gold standard for immunohematology testing, has significant limitations. DNA analysis affords a useful addition to the arsenal of methods used to resolve complex serologic investigations. This report discusses the interpretation of results obtained by DNA analyses and their correlation with serologic results. Some current applications to resolve serologic problems encountered in patient testing are reviewed and case studies are presented to demonstrate the power of combining DNA analysis with hemagglutination. ***Immunohematology* 2008;24:180–190.**

Key Words: DNA, DNA-based assays, blood group genotyping, molecular analysis, prediction of blood groups

The primary goal of patient testing performed in immunohematology laboratories is the facilitation of safe blood transfusions. Traditionally, hemagglutination-based methods have been used for this purpose as well as for routine tests including antibody identification and antigen typing of patients and donors. Hemagglutination requires the use of specific antibodies that are human source polyclonal antibodies or human or murine source monoclonal antibodies. Hemagglutination is simple, quick, and relatively inexpensive, and when carried out correctly has a specificity and sensitivity that is appropriate for most testing. However, hemagglutination, which is a subjective test, has limitations: it can be difficult to type RBCs that are coated with IgG or those from patients who have been recently transfused; it gives only an indirect indication of hemolytic disease of the fetus and newborn (HDFN) and cannot reliably determine zygosity of, for example, *RHD*. Furthermore, hemagglutination requires the availability of specific, potent antibodies, but licensed reagents are often limited in volume, extremely expensive, or not available. Finally, some antibodies are only weakly reactive, making it difficult to type patients or donors by hemagglutination.

The genes of all but one of the blood group systems (P awaits definition) have been cloned and sequenced, and the molecular bases of most blood group antigens and phenotypes have been defined. Therefore, DNA-based analyses can be used in the clinical laboratory to overcome the limitations of hemagglutination and to improve patient care. The purpose of this report is to illustrate the value of DNA-based assays for patient testing, to familiarize the reader with the interpretation of results obtained from such assays, to correlate them with serology, and to discuss discrepancies that may be observed between genotype and phenotype.

DNA-Based Assays to Predict Presence or Absence of Red Cell Antigens

Numerous genetic mechanisms generate blood group antigens and phenotypes; these are outlined by Reid.[1] Most antigens and phenotypes result from a single nucleotide change, and simple DNA-based assays can be used to predict the presence or absence of antigens on RBCs. A major advantage of DNA-based assays is that special antisera are not required and that the materials for the assays are not from human sources but can be readily purchased.

Hemagglutination is performed on RBCs and plasma or serum. Mature RBCs no longer contain a nucleus and thus cannot be used as a source of DNA. Suitable sources of genomic DNA are WBCs harvested from peripheral blood samples, a buccal smear, or urine sediment.

Fundamental to DNA-based assays is PCR amplification. This in-vitro method is used to replicate (or amplify) a particular piece (or sequence) of DNA (the target sequence). The amplified DNA sequence (amplicon) can then be analyzed by a variety of manual, semiautomated, or automated methods including allele-specific PCR, PCR-restriction fragment length polymorphism (RFLP), multiplex assays, real-time PCR, and microarray technology. The end point of most manual assays is visualization of the PCR product in a gel system after electrophoresis. With automated methods (e.g., microarray technology) the PCR products are labeled with fluorescent dyes, and the fluorescence intensity is translated by computer software to identify alleles and determine genotype. These methods are described in detail by Hue-Roye and Vege.[2]

Interpretation of Results

Hemagglutination-based testing determines the presence or absence of an antigen directly through agglutination, or lack thereof, when antibody and RBCs are combined. Reagent variability and variation in strength of antigen expression can confound the interpretation of results, particularly those that are weak. Interpretation can be subjective, and a reaction considered weak by one technologist may be considered to be negative by another. DNA-based assays test for the presence or absence of a nucleotide or a sequence of nucleotides within a gene and thus are an indirect method of predicting the likely presence or absence of an antigen. Such assays are but a snapshot of a gene at a single location, and although referred to as genotyping, the tests sample only a small portion of any gene.

Silenced or Non-expressed Genes

A single DNA-based assay may not always be sufficient. Although a gene may be detected, there are times when the gene product is not expressed on the RBCs because of the presence of a mutation that silences the gene. If a grossly normal gene is detected in a patient but the gene is not expressed, the patient could produce an antibody if antigen-positive blood is transfused. To avoid misinterpretation, when feasible, routine assays must include appropriate tests to detect a change that silences gene expression. For example, in the Duffy blood group system, a single nucleotide change (–33t>c) within the promoter region (GATA box) of *FY* prevents transcription of *FY*A* or *FY*B* in RBCs but not in other tissues.[3] Although it is rare to find silencing of *FY*A,* silencing of *FY*B* is frequent in some populations. For example, in persons of African descent, homozygosity of the –33t>c change in *FY*B* results in the Fy(a–b–) phenotype that has a prevalence of 60 percent or higher in this population.[3] When the assay is to predict the presence or absence of D, particularly in populations of African descent, it is essential to include a test for the *RHD* pseudogene that, when present, prevents expression of D on the RBC surface.[4] If the assay is for *GYPB*S* (S antigen), additional testing must be performed to detect a C>T change at nucleotide 230 in *GYP*B* exon 5 or a change in intron 5 (+5g>t); both changes prevent expression of S on the RBC surface.[5]

Controls for DNA-Based Assays

Like hemagglutination, DNA-based assays require the use of control samples. For manual methods, each assay should include control DNA samples from persons who are known to be homozygous or heterozygous for the allele of interest, but in some rare situations, samples that are homozygous for an allele may not be available. In contrast to controls for hemagglutination tests, in which the positive control is usually selected for the weakest expression of the antigen, that is, a single dose of the antigen, DNA-based assays include both homozygote and heterozygote controls for comparison of band size and location. The controls monitor PCR performance, amplification efficiency, and enzyme treatment in the case of RFLP analyses. For automated technology, generally, signal intensity of fluorescently labeled PCR products is measured by computer software and translated into presence or absence of a particular allele; the fluorescence intensity is also the basis for determining allele zygosity. The specific controls required for the various automated platforms are beyond the scope of this article. However, essential to both manual and automated assays is a water control; this is a blank that contains all reagents except DNA, the DNA being replaced by the same volume of water. The water control monitors for contamination of the test system by extraneous DNA.

Manual Assays Commonly Used

The assays outlined are those commonly used for manual testing of DNA. Automated platforms, more recently developed and summarized by Avent,[6] were not applied to the case studies described and are beyond the scope of this discussion.

PCR-RFLP

In hemagglutination, enzymes (mainly proteases) are valuable tools. They are used to cleave amino acid sequences within protein molecules to gain insight into the nature and location of a particular antigen. The enzymes used for DNA analyses are restriction endonucleases, that is, they digest or cut nucleic acids. They are commonly referred to as restriction enzymes. There are many restriction enzymes; each recognizes a specific sequence of DNA and will cut (or cleave) DNA whenever the particular sequence is present.

An example of a PCR-RFLP assay, using the restriction enzyme *Ban*I, is shown in Figure 1. An

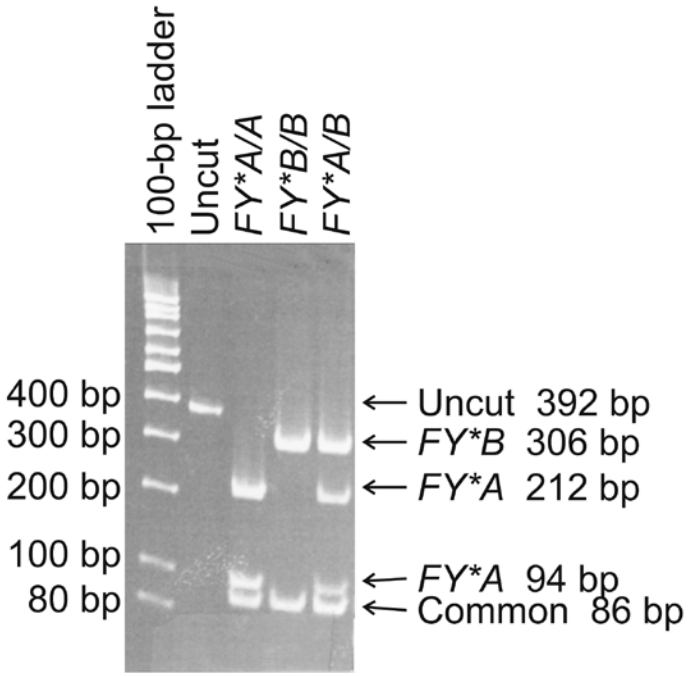

Fig. 1. PCR-RFLP assay. Digestion with the restriction enzyme *Ban*I will discriminate between two alleles, *FY*A* and *FY*B*. *FY*A* will be cleaved in two positions, resulting in three fragments, whereas *FY*B* will be cleaved in one position, resulting in two fragments. Reactions are run on an 8% polyacrylamide gel. (Lane 1) DNA ladder; (lane 2) uncut DNA in which the amplicon is not digested with *Ban*I; (lanes 3–5) PCR products digested with *Ban*I.

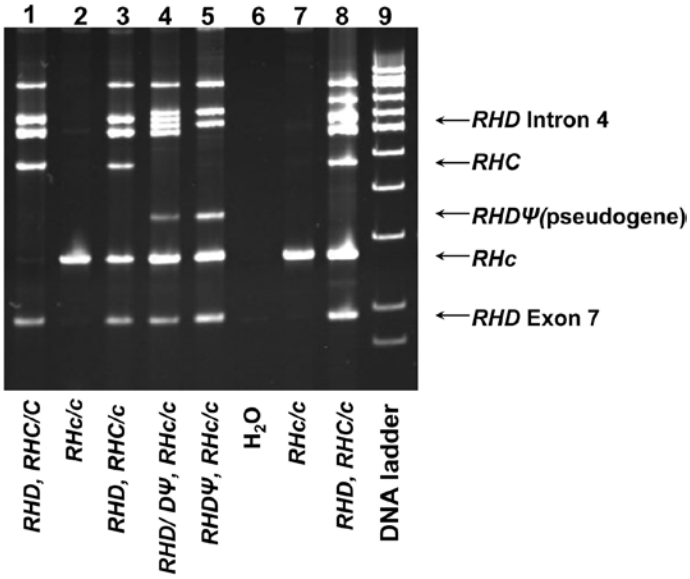

Fig. 2. Multiplex PCR analysis for *RH*. Multiplex reactions through the simultaneous amplification of sequences with five pairs of primers, run on an 8% polyacrylamide gel. (Lane 9) DNA ladder; (lane 6) water control containing no DNA; (lanes 1–5, 7, 8) PCR products representing various Rh genotypes. The DNA ladder consists of assorted DNA with a defined number of base pairs of DNA and is a basis for comparison of the DNA fragments being analyzed.

RFLP procedure distinguishes two or more alleles by virtue of a restriction enzyme site present in one allele but not the other. The specific bands from the test samples are compared against the test controls and the DNA ladder. Further detailed descriptions of these assays can be found in this issue.[2]

Allele-Specific PCR

Allele-specific PCR (AS-PCR) is most often used when the nucleotide sequence of interest in either allele is not associated with a restriction enzyme cleavage site. Two allele-specific primers that differ by a single nucleotide complementary to the nucleotide of interest are used in separate reactions, along with a common gene-specific primer (to validate the overall assay system). An internal control is included that is designed to always give reactivity. For the allele-specific band, amplification denotes the presence of an allele and lack of amplification denotes the absence of an allele. For an example of AS-PCR see Figure 2 in Hue-Roye and Vege.[2]

Multiplex PCR

A multiplex assay provides the capability of concurrently testing multiple alleles on one sample from one person. From a single reaction tube, which contains a master cocktail of multiple primers and the test DNA, multiple alleles can be distinguished. The PCR product is loaded onto either a polyacrylamide or agarose gel, and the various alleles can be discriminated by the size (and position in the gel) of the bands detected. Figure 2 shows the results of a multiplex PCR assay for *RH* used in our facility. This *RH* multiplex PCR assay uses a master mix cocktail consisting of five pairs of primers to distinguish five different alleles coded by *RHD* and *RHCE*.[4]

Applications of DNA-Based Assays

Because the molecular bases of most blood group antigens are known, it is now possible to apply DNA-based assays to overcome many limitations of hemagglutination (Table 1).

Testing of Fetal DNA

The first application of DNA-based assays for the prediction of blood group phenotype occurred in the prenatal setting and was reported by Bennett et al.,[7] who tested fetal DNA for the presence of *RHD*. Hemagglutination, including determination of antibody titer, provides only an indirect indication of the

Table 1. Applications of DNA-based assays for patient testing

Predict the RBC phenotype when • A patient was recently transfused • RBCs are coated with IgG (DAT+) and direct agglutinating antibodies or murine monoclonal antibodies are not available; or when methods to remove IgG coating are unsuccessful • A fetus is at risk for HDFN • Antibody is weak or not available, e.g., anti-Do^a, anti-Js^a, anti-V
Determine zygosity, particularly *RHD*
Resolve discrepancies, e.g., A, B, D, C, c, e
Distinguish allo- from autoantibodies
Detect weakly expressed antigens (e.g., Fy^b with the Fy^X phenotype); when patient is unlikely to make antibodies to transfused antigen-positive RBCs
Identify molecular basis of unusual serologic results
Aid in the resolution of complex serologic investigations

risk and severity of anemia or HDFN. Because of the clinical significance of anti-D, *RHD* is probably the most frequent target gene, but DNA-based assays can be used to predict the antigen type of the fetus for many antigens. When a fetus is predicted to be antigen-negative, the need to aggressively monitor the pregnancy, and any associated risk to the fetus, is avoided. When the implicated IgG antibody in the maternal circulation is not anti-D, it is still prudent to test the fetal DNA for *RHD* to preempt unnecessary requests for D– blood for intrauterine transfusion; this is particularly relevant to avoid the use of rare r′r′ or r″r″ blood when dealing with anti-c or anti-e as the implicated antibody.

Most commonly, amniocytes, harvested from amniotic fluid, are the source of fetal DNA. Chorionic villus sampling and cordocentesis are not favored because of their more invasive nature and associated risk to the fetus. A noninvasive sample source is the cell-free fetal DNA that is present in maternal plasma as early as 5 weeks of gestation; the amount of DNA increases with gestational age, and reliable results in DNA-based assays are obtained from about 15 weeks' gestation. This method has great potential, but because of patent issues, assays that use cell-free fetal DNA currently cannot be used in the United States. Regardless of the source of fetal DNA, controls (e.g., Y chromosome markers if fetus is male or polymorphic paternal markers if female) are essential to ensure that fetal, and not maternal, DNA is being tested, and systems must be in place to avoid false-negative results such as may be caused by low levels of fetal DNA. To aid in the interpretation of results, ideally, parental DNA should be tested in parallel. A more detailed discussion of fetal blood group genotyping is provided by Denomme and Fernandes.[8]

DNA-Based Assays to Predict the RBC Phenotype of Patients

Patients Whose RBCs Are Coated With IgG (DAT+)

It can be difficult or impossible to type RBCs that are coated with IgG and hence react in the direct antiglobulin test (DAT). Methods such as treatment of the RBCs with chloroquine diphosphate or EDTA-glycine acid (EGA) may be used to remove the RBC-bound IgG. These methods are not always successful, or the antigen of interest may be denatured by the treatment (e.g., EGA destroys antigens of the Kell blood group system), and direct agglutinating or murine monoclonal antibodies for the antigen of interest may not be available. Under these circumstances, DNA-based assays are a valuable tool for the prediction of the RBC phenotype and can be used to overcome the limitations of hemagglutination.

Recently Transfused Patients

For the recently transfused patient, obtaining an accurate phenotype can be a challenging and sometimes impossible task. Attempts to separate transfused donor RBCs from those of the patient are often not successful, and so the patient's most probable phenotype may be derived by the best-guess method, that is, the patient's phenotype is based on the strength of agglutination (in a mixed field reaction), the number of RBC components transfused, the time since the last transfusion, the estimated blood volume of the patient, and the prevalence of the antigen. Reid et al.[9] reported that DNA-based assays can be used to predict a patient's phenotype and that transfused donor leukocytes do not affect the result. They showed that the results of analysis of DNA extracted from peripheral blood leukocytes, urine sediment, or buccal smear were concordant with those obtained in pretransfusion hemagglutination tests, and that the best-guess method is unreliable. In our laboratory we frequently receive requests to perform DNA-based assays to help in the resolution of transfusion problems when hemagglutination cannot provide an answer. An example, which demonstrates the value of DNA-based assays for patient care, is briefly presented in Table 2. Traditionally, when a patient with autoimmune hemolytic anemia has been transfused before establishing the patient's RBC phenotype for

Table 2. Case study to demonstrate the value of DNA-based assays to predict the RBC phenotype in the care of chronically or recently transfused patients

A 59-year-old female with autoimmune hemolytic anemia and a history of chronic transfusion had made anti-E and anti-K. All E– K– RBC units are incompatible.
The patient's RBC phenotype could not be determined by hemagglutination.
No additional alloantibodies were demonstrated by repeated allogeneic adsorptions with R_1R_1, R_2R_2, and rr RBCs performed at the referring laboratory; however, the patient consistently has overt posttransfusion hemolysis.
Patient's sample was submitted for DNA-based assays.
Her probable genotype was found to be: *RHD, RHCE*C/c, RHCE*e/e, KEL*2/2, JK*A/A, GYPB*S/s, FY*A/B* (with a wild-type GATA box).
Her most likely predicted RBC phenotype is: D+C+E–c+e+, K–k+, Jk(a+b–), S+s+, Fy(a+b+).
In addition to being E– and K–, the patient's RBCs are predicted to be Jk(b–).
Did alloanti-Jk^b cause posttransfusion hemolysis? Highly likely; patient was successfully transfused with E– K– Jk(b–) RBC units without overt posttransfusion hemolysis.

minor antigens, time- and resource-consuming allogeneic absorptions will be required to determine the presence or absence of alloantibodies underlying the autoantibody. Establishing the patient's most probable phenotype through DNA-based assays will allow for matching the antigen profile of the absorbing RBCs to that of the patient, thereby reducing the number of cell types required for absorption.

DNA-Based Assays to Aid in the Resolution of Complex Serologic Investigations

DNA-based assays are an exciting new tool in our arsenal of procedures to use when attempting to resolve complex serologic investigations. A serologic investigation can be complex for a number of reasons: it may involve a combination of alloantibody and autoantibody, it may involve an antibody to a high- or low-prevalence antigen, it may involve an antibody to an antigen for which RBC samples on antibody identification panels are not typed (e.g., Do^a, Do^b, Hy, Jo^a), or it may be an investigation that is difficult to resolve with standard methods and available resources. The next section presents cases that were tested (and resolved) in our laboratory because of the power of combining hemagglutination with DNA-based assays.

To Distinguish Alloantibody from Autoantibody

A sample, with a hemoglobin value of 7.1 g/dL, was received from a 65-year-old white female with a tumor and a history of anemia. Three months earlier the patient's antibody screen had been negative, all units crossmatched had been compatible, and she had been transfused with two RBC units. Serum from the current sample reacted with all samples on an antibody identification panel.

Patient's RBCs. The results of the DAT indicated that the patient's RBCs were coated with IgG (1+ reaction with anti-IgG) and the C3 component of complement. When antigen typing was performed with monoclonal antibodies, surprisingly her RBCs appeared to be K–k–. There was no evidence for the presence of transfused RBCs. The patient's RBCs were treated with chloroquine diphosphate to remove the IgG coating (EGA, because it destroys Kell antigens, could not be used) and were found to be K–$k+^w$/–, Kp(a+^{w}b+^{w}/–), and Js(b+^{w}/–). In this situation, it was difficult to interpret the significance and validity of such findings.

Patient's Serum. By the IAT all RBCs, except those that were K_0 or Kp(b–) or treated with DTT, were agglutinated. This suggested the presence of anti-Kp^b. It is not unusual for anti-Kp^b to be an autoantibody and to be associated with the transient suppression of Kell antigens on the RBCs of the antibody maker. However, people with a genetic Kp(a+b–) phenotype who make alloanti-Kp^b can also have reduced expression of Kell antigens because of the *cis*-modifier effect of Kp^a. Therefore, the question to be answered was whether the antibody in the serum of the patient was allo- or autoanti-Kp^b.

Eluate Prepared From the Patient's RBCs. This appeared to contain autoanti-Ku because it reacted weakly with Kp(b–) and moderately with Kp(b+) but did not react with K_0 RBCs.

To gain insight into the cause of the Kell antigen suppression on the patient's RBCs, PCR-RFLP analysis of *KEL* was performed. The restriction enzyme *Nla*III was used for PCR-RFLP analysis of *KEL*3/KEL*4* (*Kp^a/Kp^b*) and revealed apparent homozygosity for nucleotide 961T, in exon 8 of *KEL.* No other changes in the patient's KEL genes were found. Based on this result, the patient's RBCs are predicted to be Kp(a+b–) and the anti-Kp^b in her serum is most likely alloantibody. The reduced expression of Kell antigens on her RBCs can be attributed to the *cis*-modifier effect of Kp^a and not to temporary antigen suppression associated with autoantibody formation.

As a Tool to Aid in the Identification of a Weakly Reactive Antibody to an Antigen for Which RBCs Panel Cell Samples are not Typed

A 73-year-old white male with hemolytic anemia (Hb = 8.2 g/dL, Hct = 24.7%) was scheduled for transfusion. His plasma was known to contain anti-c and -E, and he had been transfused with two c– E– RBC units 1 month earlier. RBCs from his current sample did not react in the direct antiglobulin test, and almost no mixed-field agglutination was observed. His plasma was now incompatible with all c– E– units, but the autologous control was negative. Anti-k was identified in his plasma. However, additional very weak reactions were observed with two examples of phenotype-matched K+k– panel cells (one sample reacted by PEG IAT, the other, which was pretreated with papain, also reacted by the IAT). It was difficult to interpret the significance of these reactions, but both of the reactive panel cells were Do(a+) whereas some of the nonreactive ones were known to be Do(a–), and the possibility that the patient had also made anti-Do^a was investigated. Because most examples of anti-Do^a and anti-Do^b are weakly reactive, available in small volume, and usually found in plasma containing other alloantibodies (as well as needing to overcome ABO incompatibility), and because the patient was transfused, DNA-based analysis was favored over hemagglutination. DNA was extracted from a peripheral blood sample from the patient, analyzed for *DO*A* and *DO*B,* and found to be *DO*B/DO*B.* Thus, the patient's RBCs would be predicted to be Do(a–b+). To determine the presence or absence of anti-Do^a in plasma that contains anti-k (and anti-c and anti-E) can be a daunting task, but fortified by the knowledge that the patient at least had the potential to make anti-Do^a, the investigation was continued. Few k– RBC samples, typed for Do^a and Do^b, were available, and the reactions observed with Do(a+) RBCs were too weak to be reliable; therefore, the patient's plasma was adsorbed onto a reactive Do(a+), k–, c–, E– RBC sample. An acid eluate prepared from the sensitized RBCs was nonreactive by PEG IAT but was shown to contain anti-Do^a by testing papain-treated Do(a+b–) RBCs by the IAT; RBCs expressing a single dose of Do^a (Do[a+b+]) did not react.

Anti-Do^a and anti-Do^b, although generally weakly reactive, are clinically significant, and antigen-negative blood should be used for transfusion. In the past, the scarcity of potent antibodies and lack of licensed reagents with anti-Do^a (or anti-Do^b) specificity forced reliance on the crossmatch to select units suitable for transfusion in similar situations. The advent of DNA-based assays has made it possible to test selected antigen-negative donors (in this case, those that are k–, c–, E–) for *DO*A* and *DO*B* and provide donor units that are predicted to lack the offending antigen. Such applications have demonstrated that testing DNA for the Do^a/Do^b polymorphism (and for changes associated with a lack of Dombrock high-prevalence antigens) clearly surpasses hemagglutination for antigen determination.[10]

As a Tool to Aid an Investigation That is Difficult To Resolve with Standard Methods and Available Resources

A sample from a 72-year-old Italian woman was received for antibody identification. The patient had presented in the emergency room feeling dizzy and unwell. Ten days earlier she had undergone coronary artery bypass graft surgery. Although the patient had a history of three pregnancies, in preoperative tests the antibody screen was negative and all units were crossmatch compatible. She received four units of RBCs during surgery, and at the time of her arrival in the emergency room her hemoglobin was 7.2 g/dL and all RBC units were incompatible when crossmatched with her serum. Surprisingly, the results of antigen typing of her RBCs suggested an absence of transfused RBCs in her circulation.

Testing of the patient's serum indicated the presence of an antibody directed at a high-prevalence antigen that was resistant to treatment with papain, trypsin, α-chymotrypsin, and DTT. RBCs lacking high-prevalence antigens that matched this profile were tested with her serum, and all, including Wr(a+b–) RBCs, reacted strongly. However, ENEP– and ENAV– RBCs were only weakly reactive, and RBCs with the M^{k}M^k, GP.Hil/GP.Hil (Mi.V/Mi.V), and GP.JL/M^k (Mi.XI/M^k) phenotypes and her own RBCs did not react. These findings indicated that the antibody detected an antigen related to the MNS blood group system, but testing for the known high-prevalence MNS antigens did not reveal the specificity. As the antigen was resistant to treatment with papain, trypsin, and α-chymotrypsin, its most likely location on glycophorin A (GPA) was close to the RBC membrane (Fig. 3).

The patient's RBCs were M+N–, S+s–, and Wr(a–) and found to be Wr(b+^{w}) when tested with eight examples of monoclonal anti-Wr^b. This altered expression of Wr^b was consistent with the possibility

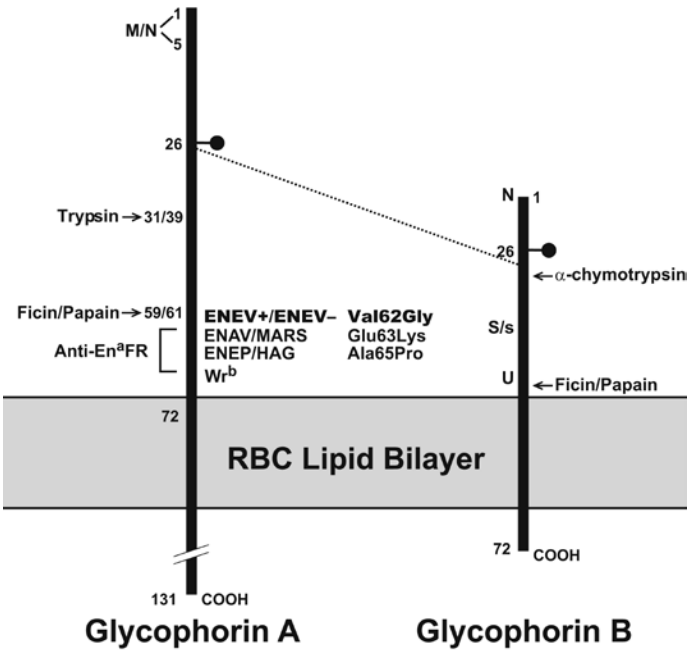

Fig. 3. Diagram of glycophorin A (GPA) and glycophorin B (GPB). Shown are the cleavage sites for proteases and the area on GPA that interacts with band 3 (Diego) for expression of Wr^b. Also shown is the location of ENEV (MNS45) on GPA. ENEV (62) is one amino acid from ENAV (63) and three from ENEP (65), which explains the weakened reactivity of the patient's serum with ENEP– and ENAV– RBCs.

that the antigen was located on GPA close to the RBC membrane, because expression of Wr^b, which is carried on band 3 and is an antigen of the Diego blood group system, is dependent on the interaction between band 3 and amino acid residues 59 to 76 of GPA.

GYPA encodes GPA and exon 4 of *GYPA* encodes the portion of GPA closest to the RBC membrane; therefore, DNA sequencing of exon 4 was performed. A change of T>G at nucleotide 242 was identified; this would be predicted to result in a Val62Gly amino acid change in GPA. In that case the antibody made by the patient detects an antigen, which was named ENEV (MNS45) by the ISBT Terminology Committee,[11,12] that requires valine at residue 62 to be expressed. The predicted location of ENEV on GPA is close to that of ENEP and ENAV (Fig. 3), and so it is not surprising that the anti-ENEV in the patient's serum gave weaker reactions with ENEP– and ENAV– RBCs. The weakened expression of Wr^b on the patient's RBCs can also be explained by the Val62Gly change as it is within the sequence of amino acids (residues 59 to 76) that interacts with band 3 to form Wr^b.

The 242T>G change ablates a DNA cleavage site for the restriction enzyme *Rsa*I. This allows for the development of a PCR-RFLP assay that not only can be used to confirm that the patient is indeed homozygous for nucleotide 242G but also can be used in place of DNA sequencing to identify other patients who may lack this high-prevalence antigen and as a screening tool (in the absence of serum) to look for potential ENEV– donors, as the clinical significance of anti-ENEV was clearly demonstrated by this patient.

As a Tool to Identify Antibodies Detecting Cromer Blood Group System Antigens

The Cromer blood group system currently contains 12 antigens of high prevalence and 3 antigens of low prevalence; they are carried on the complement protein decay-accelerating factor (DAF; CD55). Antibodies directed at the various Cromer antigens and RBCs expressing Cromer phenotypes are generally not readily available. Those that are available are not necessarily of the ABO type appropriate for the sample being investigated. Five new antigens, all of high prevalence, were assigned to the Cromer blood group system in recent years with relative ease (by comparison to some of the earlier antigens) by combining hemagglutination and DNA-based assays. To demonstrate the power of this combined approach, the investigation that identified the CRAM antigen, the most recent to be assigned to Cromer, is outlined here.[13]

During the third pregnancy of a Somali woman who had never been transfused, her serum was found to react with all panel cells tested, but it did not react with her own RBCs. The characteristics of the antibody in the initial hemagglutination testing (Table 3) indicated that the antibody was most likely directed at an antigen in the Cromer blood group system. The woman's RBCs were Cr(a+), Tc(a+), Dr(a+), WES(b+), Es(a+), UMC+W, IFC+, GUTI+, SERF+, ZENA+, and CROV+ and all of the known Cromer high-prevalence antigens were ruled out. The only remarkable finding was that the expression of UMC on her RBCs was much weaker than that on the positive control.

The Cromer antigens are carried on one of four regions of the DAF molecule referred to as short consensus repeat (SCR) regions (or complement control protein, CCP) that are encoded by exons 2 to 6 of DAF. DNA that had been extracted from a peripheral blood sample from the woman was analyzed. Regions of DNA that included exons 2 to 6

and the flanking regions of DAF were amplified and sequenced, and a nucleotide change (740A>G) was identified in exon 6. This change was predicted to encode an amino acid change, Gln247Arg, in SCR4 of DAF (Fig. 4). Thus, DNA analyses quickly revealed the molecular basis of the antigen detected by the antibody in the patient's serum and confirmed, by locating the antigen on DAF, that the antibody was indeed directed at an antigen of the Cromer blood group system. The scarce quantities of rare RBCs and sera were conserved so as to be available for other investigations. In our laboratory, if the initial findings of an investigation indicate an antibody to a Cromer system antigen (based on the characteristics in Table 3), serologic testing is halted and DNA analyses are performed on the sample. Any findings are then confirmed serologically, providing suitable serum and cell samples are available.

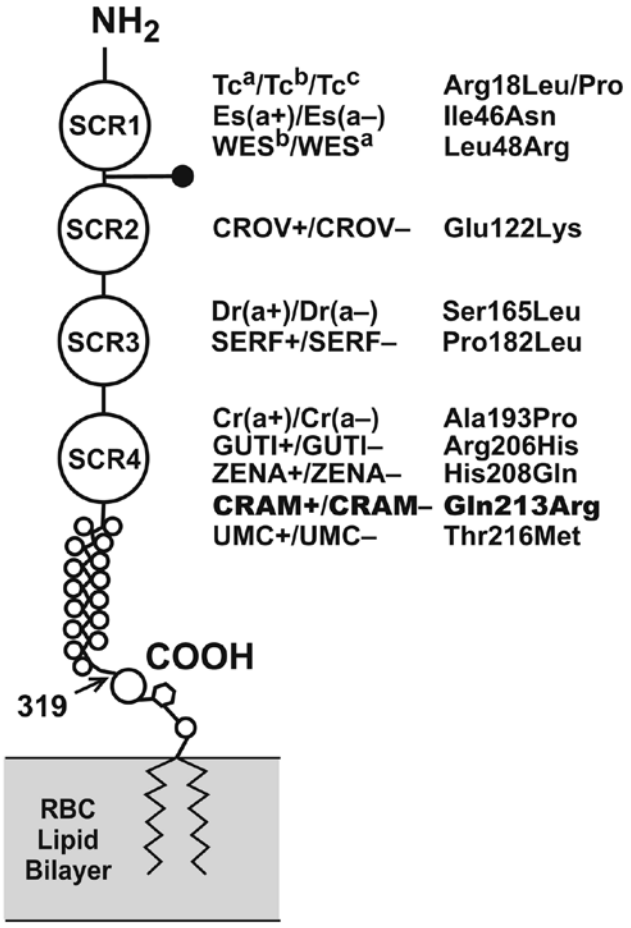

Fig. 4. Location of Cromer antigens on DAF. The amino acid associated with CRAM (residue 247) is, in the primary sequence of DAF, located between that for ZENA (residue 242) and UMC (residue 250). The change at residue 247 could explain the weakened expression of UMC on CRAM– RBCs.

Table 3. Characteristics of antibodies to high-prevalence Cromer antigens demonstrated by the antibody in the patient's serum

Reactive with all RBCs except autologous
Reactive with RBCs pretreated with ficin, papain, or trypsin
Nonreactive with RBCs pretreated with α-chymotrypsin
Weakly reactive with RBCs pretreated with 200 mM DTT; reactive with RBCs pretreated with 50 mM DTT
Nonreactive with Inab (Cromer null) phenotype RBCs
Weakly reactive with Dr(a–) RBCs
Nonreactive with PNH III RBCs
Detect antigens carried on decay-accelerating factor (DAF; CD55)

Resolving Discrepancies

The reactivity of reagents from different manufacturers with the same antibody specificity, for example anti-D, may differ when tested with RBCs expressing unusual phenotypes, such as weak or partial D. This can cause results that appear discrepant when antigen typing is performed by different facilities or even by the same facility when a different reagent is in use. With the use of DNA-based assays, it is possible to show that a typing discrepancy is attributable to a genetic variant rather than technologist error or reagent failure that could be reportable to the Food and Drug Administration.

ABO Discrepancies

From the standpoint of hemagglutination testing, the ABO system is very simple. At the DNA level, however, there is tremendous complexity and diversity, and for each phenotype many different genotypes have been identified. For this reason, it would not be practical to perform routine ABO typing by DNA analysis; it is, however, an extremely valuable tool for the resolution of typing discrepancies. It allows, for example, the distinction between an acquired phenotype and one that is inherited, such as acquired B phenotype or ABO antigen loss as a result of leukemia or malignancy.

Distinction between Weak D and a Partial D Phenotype

D is second only to the ABO antigens in clinical significance. Many D variant alleles have been defined at the molecular level.[14–17] Of particular importance from a clinical standpoint is the ability to distinguish a weak D from a partial D phenotype. An individual with a partial D phenotype can make an alloanti-D, whereas individuals with a weak D

phenotype do not. It is often difficult to differentiate these phenotypes serologically.

Determination of Rh Phenotypes That Cannot be Easily Distinguished by Serology

Similarly, there are many other Rh phenotypes that are difficult to distinguish or even define purely by serologic methods. The use of DNA-based assays facilitates the definition of many D and e variant antigens. For patients who require chronic transfusion support, such as those with sickle cell disease, and whose RBCs express an unusual Rh phenotype, RBC transfusions can pose a challenge once the patient becomes alloimmunized. To provide ongoing transfusion support for these patients, the hope is to be able to precisely match donor and patient at the genotype level. It is difficult, for example, because of a lack of potent, monospecific antisera, to type patients and donors for hr^S. Several genotypes have been associated with the hr^S- phenotype, and the use of DNA-based assays may provide a better match between patient and donor than would hemagglutination. (This is discussed in detail, with reference to Rh, by Westhoff in this issue.[18])

Weak Expression of an Antigen

DNA analysis is useful for the detection of weakly expressed antigens. For example, a patient with the Fy^X phenotype (*FY*A/FY*X*) and a greatly reduced expression of the Fy^b antigen, caused by a change at nucleotide 265, at the *FY*B* locus, is unlikely to make anti-Fy^b if transfused with Fy(b+) RBCs.[19] In this situation, DNA analysis can help determine which phenotypically antigen-negative patients can safely receive antigen-positive RBCs. The weakened Fy^b antigen attributable to the Fy^X phenotype is difficult to detect serologically with most commercial anti-Fy^b reagents. Occasionally, a reagent that detects the Fy^b of Fy^X is available. Using one of them can cause discrepancies with historical phenotypes. In such a situation DNA analysis is the more definitive assay.

Limitations of DNA-Based Assays

Correlation with Hemagglutination

Many genetic events, such as the silencing of genes discussed earlier, can cause apparent discrepancies between the results obtained by DNA analyses and those obtained by hemagglutination; some examples are shown in Table 4.[20–22]

Table 4. Examples of apparent discrepancies between DNA analysis and hemagglutination

Genetic Event	RBC Phenotype
Premature stop codon	D–, K_0, Rh_{null}
Insertion of nucleotides	Co(a–b–)
Gene rearrangement	Many phenotypes in the Rh and MNS system, e.g., D–, DVa, Mi(a+), Vw+
Presence of modifying gene	Jk(a–b–), Lu(a–b–)
Altered splice site	Jk(a–b–), Gy(a–)

Clinical Situations

DNA and hemagglutination test results may not agree in certain clinical situations that include recent transfusion, allogeneic stem cell transplant, and natural chimerism. Allogeneic stem cell transplant and natural chimerism may also cause the results of testing DNA from somatic cells (such as those obtained from a buccal smear) to differ from those of testing DNA from peripheral WBCs. When DNA-based assays are applied to the fetal-maternal setting, discrepancies may be observed if the fetal sample is contaminated with maternal DNA, if the mother is a surrogate, or if the fetus is the result of artificial insemination. Therefore, it is extremely important to obtain an accurate medical history for the patient.

Null Phenotypes

Null phenotypes for most blood group systems pose challenges for DNA-based assays. Multiple molecular bases have been identified for most null phenotypes, for example, Rh_{null}, K_0, Gy(a–), and Jk(a–b–). The identification of these null phenotypes by simple DNA-based assays sampling one nucleotide is not feasible. Several locations on a gene would need to be analyzed, and for the detection of most null phenotypes, hemagglutination is the test of choice. The majority of DNA-based assays will detect a grossly normal gene that is not expressed, and this can lead to a donor or patient being falsely identified as antigen-positive. In the case of a donor this would mean that a valuable antigen-negative (e.g., null) donor would be lost to the inventory, but such an occurrence would not jeopardize the safety of a patient receiving blood transfusion.

Conclusions

DNA analysis is an exciting new approach and a valuable tool to resolve problems in the immunohematology laboratory. But, just as hemagglutination has limitations, so do DNA-based assays. Some

limitations that were mentioned earlier in this paper are worthy of reiteration. A most important point to remember is that a genotype is not a phenotype, and there are circumstances when the allele detected at the DNA level will not be reflected by the antigen expressed on the RBC. More than one genotype can give rise to the same phenotype; this is especially so with the null phenotypes. When feasible, appropriate assays that detect changes that silence a gene should be performed. Hemagglutination testing still remains the method of choice in circumstances when the molecular basis is unknown or when multiple molecular bases exist for a particular phenotype, especially with null phenotypes. In certain situations, particularly if DNA-based analyses are used to screen donors, it is recommended that antigen-negative results be confirmed by hemagglutination using a licensed reagent when available or by crossmatching. This approach conserves expensive and rare reagents.

As discussed in this paper, hemagglutination and DNA analysis are each powerful tools in their own right, but each technology has certain inherent limitations. Their joint potential is immense when one becomes an adjunct for the other and they are jointly applied to resolve complex antibody problems and facilitate safe blood transfusion.

Acknowledgments

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References

1. Reid ME. 2007 Rock Øyen Symposium: From DNA to blood groups. Immunohematology 2008; 24:166–9.
2. Hue-Roye K, Vege S. 2007 Rock Øyen Symposium: Principles of PCR-based assays. Immunohematology 2008;24:170–5.
3. Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet 1995; 10:224–8.
4. 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.
5. Storry JR, Reid ME, Fetics S, Huang C-H. Mutations in *GYPB* exon 5 drive the S-s-U+var phenotype in persons of African descent: implications for transfusion. Transfusion 2003;43:1738–47.
6. Avent ND. Large scale blood group genotyping. Transfus Clin Biol 2007;14:10–15.
7. Bennett PR, Le Van Kim C, Colin Y, et al. Prenatal determination of fetal RhD type by DNA amplification. N Engl J Med 1993;329:607–10.
8. Denomme GA, Fernandes BJ. Fetal blood group genotyping. Transfusion 2007;47(Suppl):64–8S.
9. 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.
10. Reid ME. The Dombrock blood group system: a review. Transfusion 2003;43:107–14.
11. Lomas-Francis C, Hu Z, Hue-Roye K, et al. Novel single nucleotide change in GYPA in a person who made an alloantibody to a new high prevalence MNS antigen called ENEV (abstract). Transfusion 2006;46(Suppl):25A.
12. Daniels G, Flegel WA, Fletcher A, et al. International Society of Blood Transfusion committee on terminology for red cell surface antigens: Cape Town report. Vox Sang 2007;92: 250–3.
13. Hue-Roye K, Lomas-Francis C, Belaygorod L, et al. Three new high-prevalence antigens in the Cromer blood group system. Transfusion 2007; 47:1621–9.
14. Avent ND, Reid ME. The Rh blood group system: a review. Blood 2000;95:375–87.
15. Wagner FF, Frohmajer A, Ladewig B, et al. Weak D alleles express distinct phenotypes. Blood 2000;95:2699–708.
16. Westhoff CM. The structure and function of the Rh antigen complex. Semin Hematol 2007; 44:42–50.
17. Flegel WA, Wagner FF. Molecular genetics of RH. Vox Sang 2000;78:109–15.
18. Westhoff CM. 2007 Rock Øyen Symposium: The potential of blood group genotyping for transfusion medicine practice. Immunohematology 2008;24:190–5.
19. Olsson ML, Smythe JS, Hansson C, et al. The Fy^x phenotype is associated with a missense mutation in the Fy^b allele predicting Arg89Cys in the Duffy glycoprotein. Br J Haematol 1998;103: 1184–91.

20. Reid ME, Yazdanbakhsh K. Molecular insights into blood groups and implications for blood transfusions. Curr Opin Hematol 1998;5:93–102.
21. Reid ME. Molecular basis for blood groups and function of carrier proteins. In: Molecular and functional aspects of blood group antigens. Silberstein LE (ed). Arlington, VA: American Association of Blood Banks, 1995;75–125.
22. Reid ME. Applications of DNA-based assays in blood group antigen and antibody identification. Transfusion 2003;43:1748–57.

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The potential of blood group genotyping for transfusion medicine practice

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Molecular diagnostics is the fastest growing area of clinical laboratory medicine. The ability to rapidly amplify genes of bacterial, viral, or human origin, and the development of DNA array platforms, are driving a technology revolution in the clinical laboratory. A DNA-based testing approach is particularly applicable to blood bank and transfusion medicine for rapid, cost-effective antigen typing. Experience with DNA-based methods during the past decade has shown that these assays are reproducible and highly correlated with the RBC phenotype. The recent availability of automated, high-throughput, DNA-array platforms now moves testing from the reference laboratory setting into hospital and donor testing centers. This approach has the potential to revolutionize the process of locating antigen-negative donor units by testing for all clinically significant blood group antigens in a single assay. When partnered with the same extended typing of the patient, electronic selection of units antigen-matched at multiple blood group loci is then possible. This paper discusses the potential of this approach to improve transfusion therapy by reducing or eliminating alloantibody production in specific patient populations. These include patients facing long-term transfusion therapy and at high risk for sensitization; patients with warm autoantibodies when compatibility cannot be demonstrated by standard methods; and women for whom the production of atypical antibodies carries a risk for hemolytic disease of the fetus and newborn, or at the very least, monitoring for an at-risk pregnancy. ***Immunohematology* 2008;24:190–195.**

Key words: blood groups, DNA testing, molecular testing, transfusion practice, Rh molecular testing

Molecular diagnostics is revolutionizing the field of clinical laboratory testing. The ability to rapidly amplify genes by PCR, along with the development of high-throughput platforms and automated interpretation, is driving a technology revolution in disease and cancer diagnosis, coagulation therapy, molecular pathology, and microbe identification. DNA-based test methods are especially applicable to blood bank and transfusion medicine because the genes encoding blood group antigens are known, and the genetic polymorphisms associated with the antigens have been determined. Many blood group antigens result from single-nucleotide polymorphisms (SNP) that follow Mendelian inheritance, and the test methods target blood group genes to predict the RBC phenotype.

In the past decade, molecular testing has been limited primarily to reference laboratory environments. The manual test methods and assays are labor intensive, and discrepancies require extensive serologic and molecular genetic investigation. Significant progress has been made in validating the gene targets and investigating and explaining discrepancies between the phenotype, determined with RBCs and antibody reagents, and the genotype, determined with DNA and oligonucleotide primers. Assays that target allelic polymorphisms prevalent in all populations are reproducible and highly correlated with RBC phenotype.[1] For some blood groups, detection of common silencing mutations is also required for accurate typing. Key examples include the *GATA* mutation that silences Fy^b expression on RBCs,[2] and the two *GYPB* changes that silence glycophorin B (Ss antigen) expression.[3] For ABO and D, numerous polymorphisms are involved in antigen expression, and multiple regions of the genes must be sampled for accurate interpretation. Hence, agglutination will remain the method of choice for routine ABO and D typing for the near future. DNA analysis is a powerful adjunct to serologic testing, and specific applications for typing multiply transfused patients, typing antigens for which serologic reagents are not available, determining zygosity, testing fetal DNA, detecting weak expression of an antigen, distinguishing alloantibodies from autoantibodies, and resolving reagent typing discrepancies are discussed in the paper by Lomas-Francis and DePalma in this issue.[4]

The development of automated, high-throughput testing platforms now moves testing into the mainstream because it allows for screening of markers associated with all major antigens in a single assay without complex testing, high-level technical skills, or subjective interpretation.[5–8] Although not FDA licensed for labeling the donor unit, these platforms are a valuable screening tool to identify units negative for multiple antigens. The absence of specific antigens can then be confirmed with licensed typing reagents, and the unit can be labeled, significantly reducing the number of serologic typing reactions and the labor and reagents required to identify units of interest. Some rare units lacking high-prevalence antigens can also be identified, depending on the gene targets included on the platform design. High-throughput molecular screening will significantly impact the process of providing antigen-negative and rare donor units and is further discussed by Strauss and Reid in this issue.[9]

When partnered with testing of the patient sample, molecular screening of donors has the potential to improve transfusion therapy. Knowledge of the extended phenotype of the units in the donor or hospital inventory, when partnered with screening of the patient, presents an exciting opportunity to provide an RBC component more precisely matched at multiple blood group loci. This approach could improve patient care and transfusion outcomes by reducing alloimmunization.

Alloimmunization

Risk

Statistical analysis suggests that, overall, approximately 13 percent of patients are at risk of forming alloantibodies.[10] However, patients with sickle cell anemia have an increased risk, primarily because of the large number of donor exposures and the genetic disparity between patients of African Black ancestry and Caucasian blood donors. Additionally, patients who have made one alloantibody, indicating they are responders, are at increased risk for production of additional antibodies.[11]

The presence of blood group antibodies complicates transfusion in emergency situations and adds complexity and cost to blood bank laboratory workups. Fortunately, serious or fatal complications are not frequent, and production of RBC antibodies has been considered a manageable risk of transfusion. Alloantibodies can be of more serious consequence in some situations, particularly for patients with sickle cell disease and for pregnant women. Patients with sickle cell disease have compromised RBC survival and are anemic, so hemolysis as a result of an alloantibody could cause serious anemia and trigger a sickle cell crisis. Complications caused by alloantibodies in pregnant women can range from serious or fatal hemolytic disease of the fetus and newborn (HDFN) or, at a minimum, costly monitoring for a high-risk pregnancy. Table 1 lists patient populations for which alloantibodies are potentially of greater consequence.

Prevention

The ultimate goal to advance the practice of transfusion medicine would be to prevent alloimmunization, realizing that prevention may not be possible for all patients because of the large number of antigens (302 are known)[12] and inventory limitations, i.e., the number of donor phenotypes in inventory that potentially match the recipient. Because not all antigens are clinically significant or strongly immunogenic, an approach that focuses on the five primary systems, Rh (CcEe), Kell, Kidd ($Jk^a/^b$), Duffy ($Fy^a/^b$) and Ss, partnered with one that prioritizes patients according to alloimmunization risk, would be a first approach to prevention (Table 1). Studies to determine the degree of antigen disparity between donors and patients is needed. The data may differ significantly between geographic regions of the country, and the success of donor recruitment efforts in minority communities would impact the results. It is now possible to gather a large amount of information about the degree of antigen concordance in a community with automated genotyping. This information is the key for discussions about future possibilities for extended matching.

Table 1. Patient categories for which alloantibodies are potentially of greater consequence

Category	Antigen target for extended match
Patient with sickle cell disease (prophylactic transfusion)	C, E, K altered C, partial D, partial e Fy^a, Jk^a, Jk^b, S, s
Patient with warm autoantibodies	C, c, E, e, K Jk^a, Jk^b, Fy^a, Fy^b, S, s
Patient with alloantibody(ies)	C, c, E, e, K Jk^a, Jk^b, Fy^a, Fy^b, S, s
Women of childbearing age and female children	D, K, c

Patients with Sickle Cell Disease

Antibody production is a serious complication in patients with sickle cell disease (SCD) on long-term transfusion therapy. From 33 to 60 percent of chronically transfused patients with SCD become immunized, and transfusion is further complicated by the presence of multiple alloantibodies in as many as 45 percent of patients.[11] Alloimmunization can have severe clinical consequences, not only because there can be significant delays in finding compatible blood, but also because alloimmunization is associated with delayed hemolytic transfusion reactions (DHTR), autoantibody formation, and hyperhemolysis syndrome. Indeed, RBC autoantibody production is increasingly recognized as a serious problem concurrent with alloantibody formation, with a reported incidence of 8 to 42 percent (reviewed by Smith-Whitley[13]). The clinical significance of autoantibodies becomes secondary in the effort to honor the specific alloantibodies present, and patients receive "incompatible" blood. This is not an ideal scenario for a patient population in which the benefit is proportional to the longevity of transfused RBCs, and this may result despite an extensive laboratory workup that consumes significant time and resources.

Management of alloimmunization in SCD has been the subject of much debate,[14,15] but currently there is no standard approach. Many programs attempt to prevent or reduce the risk and incidence of alloantibody production by transfusing RBCs that are antigen-matched for D, C, E, and K. Although randomized controlled trials have not been done, this approach has been shown in single institutional and prospective multicenter experiences to significantly reduce the incidence of alloantibody production.[16–18] A 40 to 90 percent reduction was seen, depending on the extent of antigen matching (4 to as many as 15 antigens), accompanied by decreased DHTRs compared with historic rates in SCD patients.

In contrast to prophylactic antigen matching, some institutions perform phenotype matching after the patient develops the first alloantibody. Others argue that the data supporting a reduction in DHTR/H are insufficient to offset the cost of the labor and resources required to perform extended matching for SCD patients.[19]

Alloimmunization despite antigen matching

Despite antigen matching for D, C, E, and K, some patients still become alloimmunized. The antibodies often have multiple and complex specificities in the Rh system, and although the patient's RBCs may type serologically as positive for D, C, or e, the antibodies have D, C, or e-like specificities.[20] Analysis of the *RH* genes in these patients reveals they encode amino acid changes in the Rh proteins, indicating the patient and donor are not truly Rh-matched. The antibodies are often identified as "autoantibodies," but the fact that the Rh proteins differ in amino acid sequence from conventional Rh suggests that these antibodies are alloimmune in nature and have the potential to compromise RBC survival.

The prevalence of *RH* alleles that encode altered D, C, and e antigens in African Black and mixed ethnic groups explains why some SCD patients become immunized to Rh, despite conventional Rh antigen matching.[20–23] *RH* genotyping is very helpful to find compatible donors for patients with complex antibodies to high-prevalence Rh antigens. Current methods for *RH* genotyping are labor intensive because many areas of the genes must be sampled, and Rh-cDNA analysis is often necessary to detect hybrid *RH* genes.[24] High-throughput *RH* genotyping platforms are under development,[25] and, when validated, will enable SCD patients who are homozygous for altered alleles, and consequently at risk for production of alloantibodies to high-incidence Rh antigens, to be readily identified. When partnered with *RH* screening of donors, *RH* genetic matching of these patients, for which there is no serologic counterpart, could potentially eliminate alloimmunization.

Warm Autoantibodies

Patients presenting with warm autoantibodies and a positive DAT often have been recently transfused. Complex investigations requiring allogeneic adsorptions are often required to determine the presence of underlying alloantibodies, and antigen typing is complicated by the presence of contaminating donor RBCs and IgG coating the cells. An extended antigen profile is an important tool to determine the antigens to which the patient can become sensitized. Reticulocyte separations and treatment of the RBCs to remove the IgG is laborious and sometimes unsuccessful. Molecular testing enables the laboratory to predict the extended RBC phenotype. Knowledge of the predicted phenotype of the donor units in inventory would allow the laboratory to select donor units predicted to be compatible in the major blood group systems. This approach has the potential to limit

alloimmunization and to reduce the number or frequency of repeat allogeneic adsorptions for patients requiring ongoing transfusion support.

Patients Who Have Made One Alloantibody

Several studies indicate that patients who have made one alloantibody are more likely to produce additional antibodies. The production of an alloantibody identifies a responder state, as evidenced by as much as a 20-fold increased risk for additional antibodies when compared with the risk of alloantibody production with the first-time transfusion event.[26] These observations suggest this group should be considered for extended-matching when prioritizing patients according to alloimmunization risk.

Female Children and Women of Childbearing Age

D status

Approximately 2 percent or more of persons with D+ RBCs have an altered *RHD* that encodes amino acid changes in the D protein that result in loss of epitopes (partial D) or a decrease in antigen expression (weak D). These variations in D expression can cause typing discrepancies and uncertainty as to the D status. Of clinical relevance, women with partial D are at risk for production of anti-D, whereas those with weak D phenotypes only rarely become sensitized. Clarifying the D status of an obstetric patient or woman of childbearing potential is important to avoid possible fetal and newborn complications related to anti-D, or, at the least, having to closely monitor the pregnancy and manage the potential risk. Severe and fatal hemolytic disease as a result of anti-D has been reported in women with a partial DVI phenotype.[27] In contrast, a weak D phenotype is rarely associated with alloimmunization risk, and no high-titer anti-D or serious complications of pregnancy have been reported in women with weak D phenotypes. Unfortunately, serologic testing cannot distinguish altered, weak, or partial D, especially in the age of monoclonal antibody reagents.[28] Altered *RHD* can be identified with *RHD* genotyping and would only have to be performed once if made part of the permanent medical record. Although women with an uncertain D status could be treated as D– for transfusion and given Rh immune globulin, clarifying the D status (rather than simply requesting D– donor units) results in responsible stewardship of the limited D– blood supply and potentially avoids unnecessary administration of a human blood product, conserving a limited resource.

Anti-K and anti-c

The production of atypical antibodies can result in a high-risk pregnancy. Ten percent of transfusion recipients are potentially exposed to K, and approximately 18 percent to c. Severe anemia and HDFN have been reported as a result of maternal anti-K or anti-c stimulated by transfusion. Anti-K is present in approximately 1 per 1000 pregnant women, and 40 percent of K+ babies of women with anti-K are affected with severe anemia.[29] Anti-c can also be of significance and was associated with 32 deaths from HDFN in England and Wales from 1977 to 1990.[30] In the United States, 8 of 55 pregnancies complicated by anti-c required fetal transfusion,[31] and pregnancies with mildly or unaffected newborns are subject to potential invasive and costly monitoring of the pregnancy. In Europe, exposure to c and K is avoided in female recipients under the age of 50, but cost constraints have been cited as a principal limitation in the United States. It is now possible to avoid alloimmunization with a cost-effective method to determine the extended antigen profile of all units in inventory.

Considerations

Automated DNA-based typing systems do not detect all blood group polymorphisms. It is essential to know which are targeted by the manufacturer. Some, but not all, rare samples null for a specific blood group system can be detected. For example, the current BioArray HEA BeadChip detects U–, U^{var}, and HY– Jo(a–) samples, but does not target any of the Jk(a–b–), i.e., JK_{null} or Gregory (Gy^a–) null. Many different genetic backgrounds are associated with null phenotypes, and the frequency of each can differ among population groups. Even if the manufacturer were to add markers for all the nulls currently known, new silent alleles are possible in any system and would go undetected. Silenced alleles will result in false-positive DNA-typing results, although false positives encountered when screening donors for antigen-negative status would not compromise patient care because the unit would not be selected. In contrast, false-positive antigen typing would be of significance for patient care if the results were used for ruling out the presence of the corresponding alloantibody in the serum. For this reason,

this author recommends DNA-based antigen typing should not be used as the sole criterion for ruling out the presence of an alloantibody in the serum.

For patient testing, turnaround time is also a consideration. Current automated systems require about 7 to 8 hours from sample DNA extraction to readout. Although this may be substantially reduced in the future, patients urgently requiring transfusion could not be given extended-matched units, unless DNA testing had been performed previously. Like serologic typing for minor antigens, DNA testing for minor antigens would only need to be performed once and become part of the patient record.

Summary

Agglutination and antibody-based technology will remain an important methodology in the blood bank. Antibody-based methods are relatively reliable, are a direct measure of antigen expression on the RBCs, and are familiar to the profession. DNA methods cannot replace serum testing for antibody detection or identification. However, the application of molecular genotyping to transfusion medicine practice has the potential to dramatically change blood bank testing by enabling electronic selection of donor units that are antigen matched for recipients at multiple blood group loci. Although production of RBC antibodies has been considered a manageable risk following blood transfusion, prevention may now be feasible with molecular genetics. This approach holds promise to improve patient care and transfusion outcomes. Although providing extended-matched RBC units may not be possible for all patients, it is now possible for some. As genotyping advances, blood inventory management systems improve, and efforts focus on recruitment of minority donors, more patients could receive prospectively extended-matched transfusion. An initial step will be to provide some degree of extended-matched RBC units for patients with SCD, and other patients who have made an alloantibody. Women of childbearing potential would also be better served by receiving K– and c-matched RBC units whenever feasible.

The challenge for the next decade is to integrate DNA-based testing into the donor center and blood bank environment, standardize methods, obtain FDA approval for labeling donor units, and enhance information systems to effectively incorporate and use this large volume of information.

References

1. Westhoff CM. Molecular testing for transfusion medicine. Curr Opin Hematol 2006;13:471–5.
2. Tournamille C, Colin Y, Cartron J-P, Le Van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet 1995; 10:224–8.
3. Storry JR, Reid ME, Fetics S, Huang C. Mutations in the GYPB exon 5 drive the S-s-U+var phenotype in persons of African descent: implications for transfusion. Transfusion 2003;43:1738–47.
4. Lomas-Francis C, DePalma H. 2007 Rock Øyen Symposium: DNA-based assays for patient testing: their application, interpretation, and correlation of results. Immunohematology 2008; 24:180–90.
5. Avent ND, Martinez A, Flegel WA, et al. The BloodGen project: toward mass-scale comprehensive genotyping of blood donors in the European Union and beyond. Transfusion 2007; 47(Suppl):40–6S.
6. 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;45:667–79.
7. Hashmi G, Shariff T, Seul M, et al. A flexible array format for large-scale, rapid blood group DNA typing. Transfusion 2005;45:680–8.
8. Montpetit A, Phillips MS, Mongrain I, Lemieux R, St-Louis M. High-throughput molecular profiling of blood donors for minor red blood cell and platelet antigens. Transfusion 2006;46:841–8.
9. Strauss D, Reid LM. 2007 Rock Øyen Symposium: Value of DNA-based assays for donor screening and regulatory issues. Immunohematology 2008; 24:175–9.
10. Higgins JM, Sloan SR. Stochastic modeling of human RBC alloimmunization: evidence for a distinct population of immunologic responders. Blood 2008;112:2546–53.
11. Rosse WF, Gallagher D, Kinney TR, et al. Transfusion and alloimmunization in sickle cell disease. The Cooperative Study of Sickle Cell Disease. Blood 1990;76:1431–7.
12. Daniels G, Flegel WA, Fletcher A, et al. International Society of Blood Transfusion Committee on Terminology for Red Cell Surface

Antigens: Cape Town report. Vox Sang 2007; 92:250–3.
13. Smith-Whitley KS. Pediatric transfusion therapy. 1st ed. Bethesda: AABB Press, 2002.
14. Wayne AS, Kevy SV, Nathan DG. Transfusion management of sickle cell disease. Blood 1993; 81:1109–23.
15. Ness PM. To match or not to match: the question for chronically transfused patients with sickle cell anemia. Transfusion 1994;34:558–60.
16. Tahhan HR, Holbrook CT, Braddy LR, Brewer LD, Christie JD. Antigen-matched donor blood in the transfusion management of patients with sickle cell disease. Transfusion 1994;34:562–9.
17. Vichinsky EP, Luban NL, Wright E, et al. Prospective RBC phenotype matching in a stroke-prevention trial in sickle cell anemia: a multicenter transfusion trial. Transfusion 2001; 41:1086–92.
18. Ambruso DR, Githens JH, Alcorn R, et al. Experience with donors matched for minor blood group antigens in patients with sickle cell anemia who are receiving chronic transfusion therapy. Transfusion 1987;27:94–8.
19. Aygun B, Padmanabhan S, Paley C, Chandrasekaran V. Clinical significance of RBC alloantibodies and autoantibodies in sickle cell patients who received transfusions. Transfusion 2002;42:37–43.
20. Westhoff CM. Rh complexities: serology and DNA genotyping. Transfusion 2007;47 (Suppl):17–22S.
21. Hemker MB, Ligthart PC, Berger L, van Rhenen DJ, van der Schoot CE, Maaskant-van Wijk PA. 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;94:4337–42.
22. Daniels GL, Faas BH, Green CA, et al. The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. Transfusion 1998;38:951–8.
23. 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;100:4223–31.
24. Vege S, Westhoff CM. Molecular characterization of GYPB and RH in donors in the American Rare Donor Program. Immunohematology 2006;22: 143–7.
25. Hashmi G, Mezokh N, Vege S, Westhoff CM. Successful discrimination of altered e antigens by DNA analysis with a RHCE BeadChip™ (abstract). Transfusion 2007.
26. Schonewille H, van de Watering LM, Brand A. Additional red blood cell alloantibodies after blood transfusions in a nonhematologic alloimmunized patient cohort: is it time to take precautionary measures? Transfusion 2006;46:630–5.
27. Lacey PA, Caskey CR, Werner DJ, Moulds JJ. Fatal hemolytic disease of a newborn due to anti-D in an Rh-positive Du variant mother. Transfusion 1983;23:91–4.
28. Westhoff CM. Review: the Rh blood group D antigen…dominant, diverse, and difficult. Immunohematology 2005;21:155–63.
29. Caine ME, Mueller-Heubach E. Kell sensitization in pregnancy. Am J Obstet Gynecol 1986; 154:85–90.
30. Klein HG, Anstee DJ. Blood transfusion in clinical medicine. 11th ed. Oxford, UK: Blackwell, 2005.
31. Hackney DN, Knudtson EJ, Rossi KQ, Krugh D, O'Shaughnessy RW. Management of pregnancies complicated by anti-c isoimmunization. Obstet Gynecol 2004;103:24–30.

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