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<th>Platelet Antigen Analysis</th>
<th>Sickle Cell Trait</th>
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</thead>
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<td>→ 32 Human Erythrocyte Antigens per sample (HEA)</td>
<td>→ Human Platelet Antigens (HPA)</td>
<td>→ Hemoglobin-S (HgbS)</td>
</tr>
<tr>
<td>→ Rh Variant Analysis (RhCE, RhD)*</td>
<td>→ Human Leukocyte Antigens (HLA-A, HLA-B)</td>
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Molecular studies of DO alleles reveal that JO is more prevalent than HY in Brazil, whereas HY is more prevalent in New York


Because of the scarcity of anti-Hy and anti-Jo\(^a\), hemagglutination typing for the Dombrock blood group system antigens, Hy and Jo\(^a\), is not feasible. The molecular bases associated with these antigens have been determined, making it possible to distinguish HY and JO from wild-type DO. This provides a tool to predict the probable phenotype of patients and to screen for antigen-negative donors. PCR-RFLP assays and a microchip assay were used to determine the frequency of HY and JO alleles in donors from Brazil and New York. DNA from random Brazilian donors, 288 by PCR-RFLP and 599 by the bead array method (BeadChip, BioArray Solutions, Warren, NJ), was tested to determine 323G/T (HY\(^+\)/HY\(^–\)) and 350C>T (JO\(^+\)/JO\(^–\)) single-nucleotide polymorphisms. In New York, 27,226 donors who self-identified as being African American were tested by hemagglutination with anti-Gy\(^a\). Nonreactive and weakly reactive samples were tested by PCR-RFLP for the same alleles as listed above. In Brazil, 30 (3.4%) of the samples were JO/DO and 13 (1.4%) were HY/DO. In New York, of the samples that had HY or JO alleles, 14 were homozygous HY/HY, 132 were heterozygous HY/DO, 13 were heterozygous HY/JO, 14 were heterozygous JO/DO, and 3 were homozygous JO/JO. These results show that in donors from Brazil, JO (30 alleles) is more than twice as prevalent as HY (13 alleles), whereas in donors from New York, HY (173 alleles) was more than five times more common than JO (33 alleles). Immunohematology 2008;24:135–137.

Key Words: blood groups, DNA testing, Dombrock, molecular basis

In the transfusion setting, antibodies to antigens in the Dombrock blood group system have caused delayed, and rarely acute, transfusion reactions. In the prenatal setting, they have caused a positive direct antiglobulin test but not hemolytic disease of the newborn and fetus.4 Antigens in the Dombrock blood group system are carried on the Dombrock glycoprotein, which is encoded by the DO gene, also known as ART4.5 The molecular bases associated with the various Do phenotypes have been determined to be caused by single-nucleotide polymorphisms.5,7 Our ability to type RBCs for Hy and Jo\(^a\) by hemagglutination has been severely limited because of the scarcity of suitable antibodies. As the molecular bases of these Dombrock blood group system antigens have been determined, the ability to distinguish HY and JO makes it feasible to predict the probable phenotype of patients and to screen for antigen-negative donors. Based on this knowledge, we used PCR-RFLP assays and a bead microchip assay to determine the relative frequency of HY and JO alleles in donors from Brazil and New York.

Materials and Methods

Genomic DNA was extracted from theuffy coat fraction from blood samples using a DNA extraction kit (QIAamp DNA Blood Mini Kit, QIAGEN, Inc., Valencia, CA). PCR was performed using the following conditions: 100 ng of each primer (synthesized by Life Technologies, Inc., Gaithersburg, MD), 200 μM of each dNTP, 2.5 mM (for nt 323 and nt 350
of DO) or 3.0 mM MgCl₂ (for nt 793 of DO), 1.0 U DNA polymerase (HotStar Taq, Qiagen), and buffer in a total volume of 50 μL. Amplification was performed in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer, Norwalk, CT) with the following profile: 95°C for 15 minutes; followed by 35 cycles of 94°C for 20 seconds; 58°C (for nt 323 and nt 350 of DO) or 62°C (for nt 793 of DO) for 20 seconds and 72°C for 20 seconds; then 72°C for 7 minutes.⁷,⁸ PCR products were analyzed by electrophoresis in 1% agarose gel. PCR-RFLP assays were performed as previously described.⁷,⁸ The sequence of primers, PCR product size, restriction enzyme used to digest each PCR-amplified product, and expected restriction fragment sizes are given in Table 1. Digested products were analyzed by electrophoresis in 8% polyacrylamide gel.

In Brazil, DNA samples from random Brazilian donors were tested by PCR-RFLP (n = 288) and by HEA bead microchip (n = 599; BeadChip, Bioarray Solutions, Warren, NJ) to determine 793A>G heterozygous alleles, 14 were homozygous HY/JO, 3 were homozygous J0/J0, and 14 were heterozygous J0 in trans to a DO*A or DO*B (Table 2).

**Results**

In Brazil, 21 (2.4%) typed as JO/DO*B, 9 (1.0%) as JO/DO*A, 5 (0.5%) as HY/DO*A, and 8 (0.9%) as HY/DO*B. Thus, of donors who had HY or JO alleles, 30 were heterozygous JO in trans to a DO*B or DO*A, and 13 were heterozygous HY in trans to DO*A or DO*B (Table 2). All donors with HY or JO alleles were Afro-Brazilians.

<table>
<thead>
<tr>
<th>Allele Combinations</th>
<th>Brazilian Donors (n = 43)</th>
<th>New York Donors (n = 176)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HY/DO</td>
<td>13 (30%)</td>
<td>132 (75%)</td>
</tr>
<tr>
<td>HY/HY</td>
<td>0</td>
<td>14 (8%)</td>
</tr>
<tr>
<td>HY/J0</td>
<td>0</td>
<td>13 (7%)</td>
</tr>
<tr>
<td>J0/DO</td>
<td>30 (70%)</td>
<td>14 (8%)</td>
</tr>
<tr>
<td>J0/J0</td>
<td>0</td>
<td>3 (2%)</td>
</tr>
</tbody>
</table>

In New York, of the samples that had HY or JO alleles, 14 were homozygous HY/HY, 132 were heterozygous HY in trans to a DO*A or DO*B, 13 were heterozygous HY/J0, 3 were homozygous J0/J0, and 14 were heterozygous J0 in trans to a DO*A or DO*B (Table 2).

**Discussion**

In this study, in the Afro-Brazilians, JO (30 alleles) is about twice as frequent as HY (13 alleles). In contrast, in African American donors from New York, HY (173 alleles) is more than five times as common as JO (33 alleles). It is likely these findings reflect that Africans brought to South America were from a different region of Africa than those brought to the East Coast of North America.

Antibodies to antigens in the Dombrock blood group system are difficult to identify, and the paucity of reliable monospecific antisera hampers studies involving the Dombrock blood group system. At least one anti-Hy has caused biphase destruction of Hy+ RBCs.⁹ Other examples of anti-Hy as well as anti-Gy and anti-Jo have caused moderate transfusion reactions (reviewed in Reid).⁸ PCR-based testing for DOA, DOB, HY, and JO alleles provides a tool to predict the probable phenotype of patients and blood donors. This is an advantage for screening a large number of donors to find those who are Do(a–), Do(b–), Hy–, or Jo(a–), a feat not possible by hemagglutination. Thus, for Dombrock typing, DNA-based assays are not only feasible, they are more reliable

Table 1. Primers used for PCR-RFLP analyses

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Uncut Size (bp)</th>
<th>Restriction Enzyme</th>
<th>Restriction Fragment Size (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DoF</td>
<td>TACCTCAGCTGCAATGCTGAGAGAGAC</td>
<td>368</td>
<td>BsrRI</td>
<td>326, 42, 268, 58, 42</td>
</tr>
<tr>
<td>DoR</td>
<td>TTAGCAGCTGAGTTXATGCTGCAGGTCC</td>
<td>(nt 793)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DoX2F</td>
<td>TCATGCAAGGCTTGAGCA</td>
<td>220</td>
<td>BsrII (nt 325)</td>
<td>120, 92, 8 (DO)</td>
</tr>
<tr>
<td>Do378R</td>
<td>AGTAAAGTCAATGAACTTGTGAGGAAAT</td>
<td>350</td>
<td>XcmI (nt 350)</td>
<td>167, 53 (DO) 220 (JO)</td>
</tr>
</tbody>
</table>

nt = nucleotide; bp = base pairs; DO = DOA or DOB.
than hemagglutination. Our findings emphasize the importance of testing populations with different ethnic backgrounds to define their DO, and other blood group, alleles.

Acknowledgments

We thank Robert Ratner for assistance in preparation of the manuscript.

References

2. Banks JA, Hemming N, Poole J. Evidence that the Gy<sup>a</sup>, Hy and Jo<sup>a</sup> antigens belong to the Dombrock blood group system. Vox Sang 1995; 68:177–82.

There are three main alleles in the ABO blood group system, A, B, and O. The former two alleles encode glycosyltransferases resulting in the wild-type A and B phenotypes, whereas the latter allele does not encode a functional enzyme owing to a frameshift polymorphism in the majority of cases. Thus the group O phenotype is the absence of A or B sugars. More than 15 years ago the O2 allele was described; this allele did not feature the usual crippling 261delG polymorphism, which up to that point was the hallmark of an allele encoding group O, but instead had several other nucleotide polymorphisms that reduced or eliminated the activity of its resulting protein. The classification of this type of allele as encoding group O has been called into question of late as some individuals with an O2 allele appear to have a weak A phenotype. Others with the same allele do not demonstrate any A antigens on their RBCs but might be involved in reverse typing discrepancies. Even within the same pedigree these alleles do not necessarily produce a consistent phenotype. This paper will summarize the detailed biochemical and population-based evidence both for and against the O2 allele’s ability to create A antigens or the absence of anti-A in plasma. Immunohematology 2008;24:138–147.

Key words: ABO, O, O2, allele, nondeletional

Introduction to the Concept of ABO Alleles

For a short period, things were simple in the nascent field of ABO molecular genetics. There were three known alleles of the ABO gene, A', B, and O, and they correlated well with the four major ABO phenotypes. It was known that all three alleles contained 1065 bases unequally divided among seven exons, and that A alleles consistently differed from B alleles at seven in-frame nucleotide residues (Fig. 1), of which four of these single-nucleotide polymorphisms (SNPs) caused amino acid substitutions in the enzyme (Fig. 2).1-3 At that time it was also known that the O allele was one base shorter than the consensus A' allele; the deletion of a G nucleotide at residue 261 (261delG) introduced a frameshift mutation and a premature stop codon such that the protein was predicted to have only 117 of the usual 354 amino acids and completely lack an enzymatic center.1 Lacking the glycosyltransferase A (GTA) ability to transfer the A-determining sugar N-acetylgalactosamine (GalNAc) or the glycosyltransferase B (GTB) activity of transferring the B-determining moiety galactose (Gal), an O phenotype clearly is essentially the absence of A or B antigens.

Thus in the early part of the 1990s, three ABO alleles produced four phenotypes. Those halcyon days were, however, the calm before the storm; shortly after the landmark description of the structure of the ABO gene by Yamamoto et al.,1 the alleles underlying various A and B subtype phenotypes were reported.
It became clear that different SNPs could be manifest in a similar phenotype. This is not surprising given that A and B subtypes are normally first detected in the blood bank by using serologic techniques that, in a relatively crude manner, determine the amount and type of A and B antigen expressed on the RBCs. A specific subtype SNP is seldom betrayed by its phenotype; however, the same SNP can sometimes give rise to different phenotypes. Thus, almost 20 years after the cloning of the ABO gene, there are many known SNPs underlying A and B subtypes, and the process of ABO genotyping is further complicated when hybrid alleles are added to the mix. In summary, the genetics of the ABO blood group is no longer simple.

But what about group O, the null phenotype of the ABO system? This is not surprising given that A and B subtypes are normally first detected in the blood bank by using serologic techniques that, in a relatively crude manner, determine the amount and type of A and B antigen expressed on the RBCs. A specific subtype SNP is seldom betrayed by its phenotype; however, the same SNP can sometimes give rise to different phenotypes. Thus, almost 20 years after the cloning of the ABO gene, there are many known SNPs underlying A and B subtypes, and the process of ABO genotyping is further complicated when hybrid alleles are added to the mix. In summary, the genetics of the ABO blood group is no longer simple.

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expected to be $B^3/O$ as the authors assumed that a $B^3/B^1$ genotype was very rare. However, on sequencing exons 6 and 7, the 261delG SNP was not found and in fact no SNPs were discovered that would have limited the translation of this new allele’s transcript. Thus, for the first time, an allele without apparent enzymatic activity that lacked the 261delG SNP had been discovered! Other perturbations from the $A101$ allele were present: 297A>G, 526C>G, and 802G>A. Remarkably, the former two SNPs are present in a wild-type $B$ allele, whereas the 802G>A SNP was novel (Fig. 1). 802G>A predicts a Gly268Arg substitution in the enzyme; amino acid residue 268 is one of the four residues that discriminate a wild-type GTA from GTB and, along with the amino acid at residue 266, it helps to confer the enzyme’s donor sugar specificity. The 526C>G SNP predicts an Arg176Gly amino acid mutation, which again is one of the four GTA versus GTB discriminating amino acids, although the amino acid at residue 176 is more likely involved in enzyme turnover rate, not in conferring donor sugar specificity (reviewed in Yazer and Palcic). The authors transfected HeLa cells with the cDNA of their newly discovered “nondeletional” $O$ allele and could only detect background levels of $A$ and $B$ antigen expression by flow cytometry.

One year later, Danish investigators Grunnet et al. gave this new $O$ allele a name. By PCR-RFLP analysis of 150 phenotypically group $O$ blood donors and laboratory volunteers, they found 11 individuals who they described as having either an “$A$ or a $B$ allele without the (261delG) deletion.” Further analysis of this allele confirmed the finding by Yamamoto et al. of a $B$ allele–related 526G, and also found an $A$ allele–related 703G (Fig. 1). This latter residue is another $A$ versus $B$ determining nucleotide. By sequencing exons 6 and 7, these authors also confirmed the presence of both the 297A>G and 802G>A SNPs that had already been described. It was now clear that this new allele was composed of both $A$ and $B$ allele sequences. The Danish investigators thus recognized that the nondeletional allele they were describing was identical to the one published a year earlier, and they named it $O^2$. As all 11 of their donors with the $O^2$ allele were heterozygous for the deletional $O^1$ allele, the authors calculated an $O^2$ gene frequency of 3.7 percent in their population. This frequency is within the range of $O^2$ allele frequencies later reported in several populations. Grunnet et al. did not perform functional assays on the $O^2$ enzyme, nor did they employ adsorption–elution to detect whether very small quantities of $A$ antigen were present on RBCs from their $O^2$ donor.

The $O^2$ allele surfaced again a few years later in studies on $A_{el}$ and $A_{x}$ subgroups. Even though it was striking to find this infrequent $O$ allele present in several of the rare pedigrees presented, there were no data to suggest that $O^2$ was able to increase the amount of $A$ antigen expression when present, a finding that was consistent with the literature at that time.

In 2000 another comprehensive study focused on both healthy blood donors and patients with gastric carcinoma. The $O^2$ gene frequency was established to be 4.2 percent and 3.9 percent, respectively, but this study was important for several other reasons. It was the first to sequence all seven exons of the $O^2$ gene, and in so doing it revealed two SNPs in exons 2 and 5 that had not previously been detected in this allele. The 53G>T SNP is interesting because it represented a polymorphism specific to the $O^2$ allele; up to this point, the $O^2$ gene appeared to be a hybrid of $A$ and $B$ alleles without any novel sequences of its own except for 802G>A, but the new data suggested that this allele had undergone its own evolution (Fig. 1). Later investigators would reveal more similarities between the $O^2$ and $A^i$ alleles in the upstream CCAAT-binding factor (CBF) enhancer region (both have only one 43-bp element as opposed to the other major alleles, which have four). Analysis of the introns of the $O^2$ allele revealed that it shares some common sequences with other $O$ alleles in introns 2, 4, and 5, and with $B$ alleles in intron 6, while also demonstrating a few unique SNPs in introns 2 through 5.

The authors of the previous studies on $O^2$ predicted that if a protein was to be translated from this gene, it should contain all 354 amino acids and it should thus cross-react with antibodies to both the wild-type GTA and GTB enzymes themselves. Indeed this latter postulate was shown to be the case; when SF9 insect cells were transfected with the full-length $O^2$ transcript, staining with an antibody that binds to amino acid sequences common to wild-type GTA and GTB revealed a Golgi pattern of localization for the $O_2$ protein. This pattern of staining was observed in most normal and neoplastic tissues from $O^2/O^2$ gastric cancer patients. So whereas the $O_2$ protein was expressed in its expected location, its functionality remained dubious. Expression constructs of both
the full-length and soluble forms of the enzyme failed to show activity above background levels with either GalNAc or Gal. Similar findings had been demonstrated in 1996 when Yamamoto and McNeill produced expression constructs of an A101 gene and mutated the amino acid at residue 268 with all 20 amino acids; when the construct featured Arg268, A antigen was not detected on the HeLa cells by flow cytometric analysis and this mutant construct failed to show GTA enzymatic activity. However, it should be noted that the other O-related SNPs were not present in this construct. When the normal and neoplastic gastric tissues from the O1O2 patients were stained for A antigens, only an equivocal amount of immuno-reactivity was observed in a few specimens. Thus in 2000, the overwhelming weight of evidence was on the authors’ side when they concluded that this allele’s designation as an O allele, despite its production of a full-length transcript whose protein localizes where functional GTA and GTB enzymes do, was valid as it did not appear to have enzymatic activity.

**Crystallographic structure of O2 protein reveals mechanism for reduced or abolished enzymatic activity**

More support for classifying O2 as an O allele came in 2005 when Lee et al. studied an *Escherichia coli*-optimized model of the soluble truncated form of the O2 enzyme (lacking amino acids 1–53). In their construct, the O2 enzyme had six orders of magnitude less ability to transfer GalNAc to the synthetic acceptor substance (H antigen) compared with the wild-type GTA enzyme. Incorporating all three amino acid mutations, the activity level (kcat) of O2 was 3.4 × 10−5 s−1 compared with the wild-type GTA activity of 17.5 s−1 with a GalNAc donor! The soluble form of O2 also had a marked increase in the binding constant for the GalNAc donor substrate (Kd) with a smaller increase for the H antigen acceptor substrate (Ka). Moreover, they showed that the principal deactivating mutation was Gly268Arg (caused by the 802G>A SNP), as revealed in the enzyme’s crystal structure; the bulky side chain of Arg268 causes steric blockade of the access of GalNAc to its binding site in the enzyme’s active center, hence the observed increase in the Ka and its resulting inability to catalyze the formation of A antigen. Given that in the clinical studies described above no A antigens were regularly detected on the RBCs or tissues of individuals with an O2 gene, and compounded with these in vitro data, it looked like the meager kcat of O2 with a GalNAc donor was basically equal to zero.

Interestingly, the 802G>A SNP turned out not to be unique to the O2 family of alleles. The dbRBC contains seven alleles that feature this SNP, of which two are not specifically identified as group O alleles. These others include Aw08 (see below), and Bw-18. This latter allele produced a Bx phenotype caused by an Ala268Thr amino acid mutation. Interestingly, the steric interference observed with the O2-specific Arg268 does not appear to occur with Thr268. Nevertheless, the kcat of the Bw-18 enzyme is reduced by more than 100-fold compared with a wild-type GTB with Gal donor, again indicating the importance of residue 268 for the enzyme’s activity.

### 2. Do O Alleles with 802G>A Give Rise to GTA Activity? Yes.

Given the results of all the investigations on the O2 allele, it was a surprise when, in 2003, two blood donors who were homozygous for nondeletional O alleles featuring the 802G>A SNP (also referred to as 003 alleles) with an RBC phenotype consistent with the A2 subgroup were described. Both donors also had weak anti-A1 and strong anti-B antibodies in their serum. One of the two donors had another type of nondeletional O allele; in addition to the previously reported SNPs for O2, this donor’s allele also featured a 488C>T polymorphism, which predicts a Thr163Met change. As the authors believed that this new nondeletional allele had some intrinsic GTA activity, they named it AB0*Aw08 (probably identical to O2-9, Fig. 1). It was hypothesized that Thr163 somehow allowed the enzyme to overcome the significant steric inhibition caused by Arg268, thus “reactivating” the enzyme and allowing it to create a very weak A phenotype. The authors did not comment on the fact that although only one donor was heterozygous for AB0*Aw08, both donors had identical RBC phenotypes. Thus even the donor who was homozygous for two standard nondeletional O alleles (O2) without the Thr163Met mutation was allegedly capable of producing what they described as an A2 phenotype. This speaks against the notion of a reactivating mutation, as does the subsequent finding of an apparently identical allele in a blood donor with a normal group O phenotype and strong anti-A and anti-A1.
Allelic enhancement might contribute to the O₂ enzyme’s GTA activity

Allelic enhancement is a phenomenon that can lead to unexpected phenotypes, typically noted when a pedigree analysis is performed.²⁴ It has been reported in the setting of two full-length products from the ABO locus; typically one enzyme is mutated and would be expected to produce a weak A (or possibly B) phenotype, whereas the second ABO glycosyltransferase (GT) is fully functional. Under most circumstances, the normal ABO GT would then outcompete the weakened enzyme and can even result in the failure to detect any of the mutated enzyme’s antigens.²⁵ Uncommonly though, greater than expected antigen production by the mutated enzyme can result when it is expressed along with a fully functional and full-length ABO GT. In the original report, A⁺ and B alleles were studied in a family, and a nearly 10-fold increase in the number of RBC A antigen sites was observed in the A⁺B family members compared with their A⁺O relatives. In addition, serum GTA activity was only detected in the A⁺B family members.²⁶ Because wild-type GTB is known to have some low-level intrinsic GTA activity, it was even more exciting when O⁺ was implicated in allelic enhancement in a Polish family; the propositus demonstrated a weak A phenotype with an A⁺⁻⁻⁻⁴/O⁻⁻⁴⁻⁻¹ genotype, while her parents both had O phenotypes and were heterozygous for A⁺⁻⁴ or O⁻⁻¹ alleles with common deletional O alleles in trans.²⁷

The biochemical basis of allelic enhancement is still unclear, although in an E. coli–optimized model of soluble O₂ proteins, dimerization appeared to occur by means of the enzyme’s stem region.²¹ Thus, there is a potential to produce more enzymatically favorable protein heterodimers leading to increased catalysis if both fully functional and less optimal ABO-GT are present in heterozygous individuals. A scenario supporting this speculation has recently been reported for other types of glycosyltransferases in which the enzymatic activity increased significantly after heterodimer formation.²⁸

RBCs from blood donors with an O² allele appear to demonstrate A antigens

Seltsam et al.²⁹ followed up their initial report of nondeletional group O alleles with limited GTA activity with a larger survey of both blood donors with a group O forward type but variably reduced levels of anti-A and normal levels of anti-B on their reverse type, samples that were referred to their laboratory because of a suspected A subtype, and in vitro expression studies of both the O² (O03) allele and the ABO*Aw08 allele. Some of the results were difficult to interpret, in particular some of the serologic findings among the 12 donors (and 4 family members) who were at least heterozygous for a nondeletional O allele. All but one had an uncomplicated group O forward type, although some demonstrated weakening of their serum anti-A₁, and some completely lacked anti-A. These reverse typing perturbations can be considered a surrogate marker for A antigen production, if not on the RBCs themselves then perhaps on some other tissue such that the immune system produces a muted antibody response to this antigen as it recognizes the low level of A antigen as a “self” antigen. In other individuals the level of anti-A as assessed by agglutination was normal. A antigens were not detectable on any of these RBCs by flow cytometry. Using both polyclonal anti-A and anti-A,B, A antigens were barely detectable on some of these RBCs using two different adsorption–elution techniques,²⁶ which is unusual because even in the weakest possible A subgroup, A⁺, the eluate generally reacts very strongly (typically 3–4+). In this study, Seltsam et al.²⁹ did not report the strength of the eluates prepared by using their anti-A and anti-A,B reagents with other A subgroup cells as a positive control, nor did they specify the results of testing the “last wash” supernatant; thus it is unclear how to interpret the very weak (often unconventionally reported as 0.5+) agglutination they observed in their eluates. Furthermore, the pattern of eluate positivity is hard to interpret; some donors showed very weak reactivity using both eluate methods and both reagents, whereas others showed reactivity only with one reagent and one eluate method. The lack of consistent eluate reactivity patterns coupled with the generally weak agglutination makes drawing firm conclusions about the GTA activity of O₂ proteins difficult in this study. In this study, one donor who was heterozygous for a nondeletional O allele apparently demonstrated macroscopically visible agglutination on forward typing using one particular monoclonal anti-A reagent (reported as 1+ agglutination using the Seraclone reagent from Biolog AG, Dreieich, Germany), yet the donor’s eluate was completely negative with both reagents and both elution techniques²⁹ This individual also featured nearly normal agglutination
of anti-\(A_1\) on reverse typing. It is difficult to reconcile these findings: there were sufficient \(A\) antigens on this individual's RBCs to cause macroscopically visible agglutination, yet anti-\(A\) could not be eluted from them, nor was any \(A\) antigen detectable by flow cytometric analysis. It must be considered that the apparent weak \(A\) activity noted in some \(O^2\) donors and with some reagents may actually represent not the true GalNAc-based \(A\) antigen but some alternative \(A\)-like structure that cross-reacts with certain antisera and sometimes downregulates the \(A\) response of the individual. For instance, it should be possible to assess whether the \(O_2\) protein is slightly better at transferring a sugar other than GalNAc to the \(H\) precursor. Interestingly, minor reactivity with alternative carbohydrate substrates has been reported with wild-type GTA and GTB.\(^{30}\)

The \(Aw08\) allele was once again detected in two donors who had \(O03/Aw08\) genotypes; the presence of the \(Aw08\) allele did not cause increased \(A\) antigen expression on their RBCs compared with those of donors with an \(O03\) allele.\(^{29}\) In the in vitro component of their experiments, anti-\(A\) was elutable from HeLa cells transfected with the \(O03\) and \(Aw08\) genes, and eluates from both of these transfections demonstrated identical agglutination strengths.\(^{29}\) It is possible that the transfection experiments do not necessarily reflect the activity of these alleles in vivo; the eluates from the RBCs of donors with nondeletional \(O\) alleles ranged from negative to weakly positive, and the strengths did not change significantly between eluates prepared from anti-\(A\) or anti-\(A,B\) reagents. In the transfection experiments, the agglutination strengths were generally much higher than those of the donors, and in all cases showed a full grade stronger agglutination with the anti-\(A,B\) reagent.\(^{29}\) As in their donor studies, Seltsam et al.\(^{29}\) did not report the results of the last wash supernatant for the transfection adsorption–elution experiments, nor was this technique originally intended to be used on HeLa cells.

In their analysis of 16 donors and family members with an \(O^2\) allele, an interesting side note emerged. Although it is difficult to compare the eluate results among donors when the strength of agglutination was so weak, there did not appear to be a difference in the diminution of anti-\(A_1\) or anti-\(A\) on reverse typing, nor an increase in strength of the eluates in donors who were homozygous for nondeletional \(O\) alleles compared with those who were heterozygous for a deletional \(O\) allele.\(^{29}\) This appears to weigh against zygosity as a contributing factor in the (possible) production of \(A\) antigen. Further evidence that homozygosity of nondeletional \(O\) alleles is not required to cause ABO discrepancies on automated typing instruments came from another German study of 2196 group \(O\) blood donors by Wagner et al.\(^{31}\) In 45 of these samples their Olympus PK7200 could not determine the blood group because of irregularities with the anti-\(A\) reverse typing, and in 38 of those cases, the donors were at least heterozygous for an \(O^2\) allele (1.7% of group \(O\) donors). Given that the expected frequency of the \(O^2\) allele in the German population is approximately 1.6 percent,\(^{29,32,35}\) it was expected that in total approximately 65 to 70 donors with at least one \(O^2\) allele should have been discovered in this study. That only 38 such donors were identified suggests that the allele frequency in that specific donor population is lower than expected, or, alternatively, it is possible that there are other as yet unidentified factors that regulate the activity of the \(O_2\) protein such that in the approximately 30 donors who were not identified their \(O_2\) protein lacked sufficient GTA activity to create an ABO discrepancy.

Curiously, two donors who were heterozygous for the \(O^2\) allele produced normal levels of anti-\(A\), yet they were enumerated among those samples in which the instrument could not interpret the ABO type because of diminished titers of anti-\(A\).\(^{31}\) In this study too, zygosity did not seem to influence the diminution of anti-\(A\) level; in the 10 donors who were devoid of anti-\(A\), all were heterozygous for the \(O03\) allele along with a deletional \(O\) allele. Three donors were homozygous for nondeletional \(O\) alleles, and they demonstrated weak (but not absent) anti-\(A\) on the reverse type, again indicating that zygosity is not likely affecting these results, and also that the Thr163Met mutation in the \(Aw08\) enzyme does not confer on it extra GTA activity compared with the \(O03 (O_2)\) protein.\(^{31}\) Unfortunately, adsorption–elution studies were not performed (Franz Wagner, personal communication) on the RBCs from the donors with an \(O^2\) allele to try to determine whether \(A\) antigens were present or the diminution of anti-\(A\) on reverse typing had another cause. Even supplying the levels of anti-\(B\) would have indicted whether the diminution in antibody levels was restricted to anti-\(A\) (as the authors suggest), or it was related to a global reduction in antibody titers.
Although the $O^2$ allele was present in the majority of donors with reduced titers of anti-A, overall the results of this study were not clear-cut; there were seven donors who were homozygous for deletional $O$ alleles who also demonstrated weak anti-A on reverse typing.\[^{31}\] Surely another explanation for the diminished reverse typing must be sought in these donors as deletional $O$ alleles cannot produce A antigens. Conversely some donors with $O03$ alleles did not demonstrate any diminution of their plasma anti-A on reverse typing, whereas one $O03$ heterozygous donor demonstrated diminution of both anti-A and anti-B on repeat testing.\[^{31}\] The diminution of anti-B is not explicable by any intrinsic GTA activity of the $O_2$ protein.

In total, these authors have interpreted their results to indicate that the members of the $O^2$ family of alleles have intrinsic GTA activity. As Lee et al.\[^{21}\] reported, the in vitro activity of the soluble form of the $O_2$ enzyme was $3.4 \times 10^{-5}$ s\(^{-1}\); some of the aforementioned results suggest that this number, although very small, is not quite equal to zero. Still, in the absence of unusual confounders it can be predicted that an A phenotype will be produced if a consensus A gene is present and a B phenotype will be produced if a wild-type B gene is present. However, the RBC phenotype cannot be predicted with a high degree of certainty when a nondeletional $O$ allele is present. This is perhaps even more evident in the presence of nondeletional $O$ alleles other than $O^2$.\[^{9,34}\]

**The weak A phenotype created by the $O^2$ allele is highly variable**

The variability of the $O^2$-associated phenotype was highlighted in a recent paper analyzing 19 $O^2$ heterozygous samples from patients and donors from around the world.\[^{9}\] Three samples produced the expected normal O phenotype on both the forward and reverse typing, 10 samples had diminished or absent anti-A in the setting of an otherwise normal O phenotype, and in the remaining 6 samples some degree of A antigen expression was detected, generally by adsorption–elution. A variety of $O^2$ family alleles (including a few new family members, i.e., $O^2$ alleles with one or two additional missense mutations) were detected among these 19 heterozygous donors, including $Awo08$, which was also found in a donor with a completely normal O phenotype as mentioned above.\[^{9}\]

An interesting family study also serves to highlight the variability of A antigen synthesis in donors with an $O^2$ allele. The male propositus had an $O^*/O^2$-2 genotype and an $A_1$ RBC phenotype. His father, who genotyped as $O^*/O^2$-2, had a completely normal O phenotype including normal-strength anti-A! Thus the same allele within the same family can behave differently.\[^{9}\]

This may be the mechanism for the GTA activity associated with the presence of $O^2$

Hosseini-Maaf et al.\[^{9}\] offered an interesting explanation for how an $O^2$ allele might demonstrate GTA activity when it is inherited with a common deletional $O$ allele; terming it "autologous chimerism," the authors postulate that if recombination or gene conversion occurs between these two $O$ alleles such that the 261delG of the deletional allele is replaced with the otherwise consensus DNA in this area from the nondeletional $O$ allele, then the mRNA from the (formerly) deletional $O$ allele will lack the premature stop codon and translation can proceed normally. This hybrid transcript would not feature the crippling 802G>A SNP characteristic of the $O^2$ family of alleles, and thus a functional hybrid A allele could be produced. As this could be a somatic mutation occurring randomly during mitosis it would not be transmitted from generation to generation. This randomness would explain why the son with the $O^*/O^2$-2 genotype produced an apparent $A_1$ phenotype, whereas his father with basically the same genotype did not. It should be noted that it still remains to be shown experimentally whether this appealing hypothesis is valid or not. The fact that other nondeletional $O$ alleles carrying early (exon 2) or late (exon 6 or 7) nonsense mutations resulting in premature stop codons behave similarly to $O^2$ in that they can produce different phenotypic variants of A expression or no A at all favors a general explanation that can be applicable to any combination of a deletional and a nondeletional $O$ allele.\[^{9,34}\]

However, autologous chimerism, the potential process of creating a trans-cis hybrid and in this case a functional A allele, would not explain the suspected GTA activity in those rare individuals homozygous for nondeletional $O$ alleles ($O^2$ is the only example so far) because identical alleles cannot overcome each other’s problematic polymorphisms. Finally, it will be interesting to see whether a future $O$ allele based on a B allele backbone results in a similar phenomenon with variable expression of the B antigen.

By the end of 2005, several types of studies had begun to describe the enigma of the $O^2$ allele: (1) studies based on samples referred to a reference laboratory because of an ABO discrepancy or suspicion of a weak A subtype phenotype,\(^9,29\) (2) studies based on blood donors whose predonation samples created ABO typing discrepancies,\(^29,31\) (3) studies of group O blood donors whose predonation samples did not feature ABO discrepancies,\(^29\) and (4) in vitro studies of the expression and activity of the $O^2$ allele.\(^13,18,20,21,29\)

As illustrated above, these studies came to different conclusions because of variable results concerning the activity and consequences of the $O^2$ allele and the encoded $O_2$ enzyme. In some cases the overall conclusion was that the $O^2$ allele was not enzymatically functional, whereas in other studies, there did appear to be both direct and indirect evidence for its weak enzymatic activity.

Yazer et al.\(^35\) took a different approach to the $O^2$ allele when they studied its incidence and properties in labeled group O units that were available for transfusion from their blood banks in Pittsburgh, Pennsylvania, and Lund, Sweden. The genotypes of these group O donors were not known at the time of their donation. These investigators assayed a total of 779 group O units for the presence of an $O^2$ allele and found 40 donors (5.1%, allele frequency 2.6%) who were heterozygous for an $O^2$ allele. None of these units had caused ABO discrepancies either on automated ABO typing instruments used for routine donor typing or when the ABO type was performed manually after these units were identified. Unlike in the previous study of blood donors with an $O^2$ allele,\(^29\) when three donors from Pittsburgh had adsorption–elution performed on their RBCs using a polyclonal anti-A reagent, anti-A was not recoverable in the eluate. The A antigen was not detected when some of these donor RBCs were subjected to a very sensitive flow cytometry assay capable of detecting the minute quantity of naturally occurring A antigen on B cells,\(^36\) nor when the plasma from the $O^2$ donors was used to upload A antigens onto group O RBCs. In fact, the activity of the plasma-borne $O_2$ enzyme from these donors was not above the water blank baseline. However, although A antigens could not be directly demonstrated on these RBCs, on average the titer of anti-A, and in particular anti-A was clearly diminished compared with that from control $O^1/O^1$ sera.\(^35\) Unfortunately, titers of other antibodies were not performed to exclude a global reduction in immunoglobulin titer, but again this appears unlikely to coincide with $O^2$ carrier status. Thus, as described earlier, the presence of A antigens on other tissues cannot be excluded.

The authors of several earlier studies that had shown some weak GTA activity of the $O_2$ protein speculated that despite the low level of A antigens expressed on these RBCs, it should be safe to transfuse them to group O and B donors who would be expected to have naturally occurring anti-A in their plasma.\(^29,31\) Yazer et al.\(^35\) provided some confirmation of this hypothesis by performing a retrospective analysis on 19 of the $O^2$ units that were transfused to recipients with naturally occurring anti-A. There were no reported immediate hemolytic events, and in six recipients for whom more detailed information was available, all six had the expected increase in their posttransfusion hemoglobin level. The biochemical markers of hemolysis were not ordered on any of the recipients after transfusion, which also indicates that the attending clinicians did not suspect hemolysis after transfusion of these $O^2$ units.\(^35\)

In a small prospective study, so far only reported in abstract form, the investigators monitored the anti-A levels in group O recipients after transfusion of RBC units from group O donors positive for an $O^2$ allele (i.e., $O03$ and $Aw08$).\(^37\) They found no changes in these levels when pretransfusion and posttransfusion samples were compared. This further supports the notion that $O^2$ units do not pose a risk of hemolysis by introducing the A antigen into group O recipients.

**Conclusions**

Whether the $O_2$ protein has consistent GTA activity remains to be seen, and the factors that regulate any intrinsic GTA activity have not been elucidated. At most, the $O^2$-encoded protein might be able to produce a very weak A phenotype, although in many cases evidence for the presence of A antigens on the RBCs is indirect, through reduced anti-A, and anti-A levels on reverse typing. In other situations individuals with an $O^2$ allele do not seem to demonstrate even a weak A phenotype, nor weakened anti-A or $-A_1$ in plasma. In the meantime, the ABO system continues to serve as a wonderful model system for polymorphic carbohydrate biology, and the study
of the O^2 protein has shed some more light on the fascinating biology of the ABO glycosyltransferases and their underlying loci.

References

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Molecular analyses of GYPB in African Brazilians

R. Omoto, M.E. Reid, and L. Castilho

The molecular background of variant forms of GYPB is not well studied in Brazilians of African descent. The present study was carried out to determine the molecular bases of the S–s– phenotype and the frequency of GYPB*S silent gene for the S–s+ phenotype in a blood donor population of African Brazilians. In this study, 165 blood samples from African Brazilians (Northeastern Brazil) who phenotyped as S–s– (n = 17) and S–s+ (n = 148) by hemagglutination were selected. Allele-specific (AS)-PCR and PCR-restriction fragment length polymorphism (RFLP) were used to identify the variant forms of GYPB. In 13 of 17 S–s– samples (76.5%), both GYPB were deleted. In 137 of the 148 S–s+ samples (92.6%), the AS-PCR was consistent with the S–s+ phenotype. In 4 of the S–s– samples (23.5%) and 11 of the S–s+ samples (7.4%), the AS-PCR showed the presence of a GYPB*S allele associated with silencing of S. In the 4 donors with the S–s– phenotype, there was homozygosity (or hemizygosity) for the GYP(P2) allele (n = 2), homozygosity (or hemizygosity) for the GYP(NY) allele (n = 1), and heterozygosity for the GYP(P2) and GYP(NY) alleles (n = 1). In the 11 donors with the S–s+ phenotype, there was heterozygosity for GYP(P2) allele (n = 8) and heterozygosity for GYP(NY) allele (n = 3). This study reports for the first time the molecular mechanisms responsible for the S–s– phenotype in a population of African Brazilians and provides new information about the frequency and molecular bases of the GYPB*S silent gene (7.4%) in this population.

Key Words: African Brazilians, S–s– phenotype, GYPB(P2), GYPB(NY), MNS blood group system, GYPB*S silent gene, blood group genotyping.

The MNS blood group system is a highly complex system that consists of more than 40 distinct antigens. These antigens are carried on glycophorin A (GPA), glycophorin B (GPB), or hybrid proteins that arise from unequal crossover, nucleotide substitution, or gene conversion events between the glycophorin genes.

The genes encoding GPA (GYPA) and GPB (GYPB) are located on chromosome 4 together with a third gene of this glycophorin family, GYPE. In addition to sequence homology and proximity between the glycophorin genes, recombination hot spots have been identified and demonstrated to generate many different hybrid GYP gene products at the RBC surface, as a result of reciprocal and nonreciprocal exchange of nucleic acids. These hybrid molecules often carry one or more novel antigens of the MNS blood group system. Furthermore, the expression of more common MNS system antigens, such as S or s, may be affected if the encoding sequence is close to the crossover site, manifested by unexpected results with some antisera. In addition, Storry et al., 5 studying the molecular bases of the weakened expression of S or s associated with the low-incidence antigens M', s3, and Mit, showed that Arg35 is important for full expression of S and Pro39 and Thr3 were also important for full expression of s.

The absence of GPB on the RBCs as a result of a GYPB gene deletion is characterized by the S–s– phenotype, 6 which is found in persons of African descent. RBCs of approximately 1 percent of African Americans, and up to 37 percent of West Africans, type as S–s–. The S–s– phenotype is associated either with the absence of the high-prevalence antigen, U, or with weakened expression of U (U+var). U is commonly found in all populations. The S–s–U– phenotype is found among Black ethnic groups and appears to correlate with the geographic prevalence of malaria infection as a result of the deletion of GYPB, whereas the S–s–U+var phenotype has been associated with two variant GPB proteins.

Storry et al. 4 reported in 2003 a large cohort study of S–s– donors to determine the molecular alterations of the S–s– phenotype and their distribution in the African American population. In this study, it was confirmed that DNA from donors whose RBCs failed to react with a potent anti-U/GPB was not amplified by allele-specific or gene-specific primers designed to detect GYPB. In contrast, donors whose RBCs were reactive with anti-U/GPB demonstrated the presence of variant forms of GYP.He (in the majority of samples) or of GYPB by allele-specific assays.
The GYP.He variant is an altered form of GPB (B-A-B hybrid gene) as a result of a gene recombinational event. The ultimate result of this event was the generation of a composite sequence defining the Henshaw (He) epitope with a concomitant abolition of the GPB-associated “N” antigen in some Africans. All these samples demonstrated the presence of the silenced GYPB*S allele. The absence of S at the RBC surface was demonstrated to be caused by a change at nucleotide 208 (G>T) and 230 (C>T) of exon 5 (named variant GYPB[N], or GYPHe[N]), or a g>t change at +5 of intron 5 (named variant GYPB[P2], or GYPHe[P2]), which led to partial or complete exon skipping. Sequencing and PCR-restriction fragment length polymorphism (RFLP) analyses showed that the GYP(P2) allele was the most common mechanism in donors and patients with the S–s–U+var phenotype. The N-terminus of glycophorins carrying either GP(P2) or GP(NY) can express “N” or He antigens.

In this study, DNA-based assays were used to identify variant forms of GYPB in Brazilian blood donors of African descent with the S–s– and S–s+ phenotypes. GYP(P2) and GYP(NY) alleles have been identified as the mechanisms for the S–s–U+var and have also explained the presence of the GYPB*S allele with silencing of S in S–s+ phenotypes in this donor population.

Materials and Methods

Blood Samples

EDTA blood samples were obtained from healthy volunteer blood donors, who self-identified their ethnicity as African Brazilians, at the Sao Rafael Hospital Blood Bank. Institutional Review Board–approved informed consent was obtained from each blood donor.

Serologic Analysis

The S/s/U status of donors’ RBCs was determined by IAT-hemagglutination in gel cards (DiaMed AG, Morat, Switzerland) using commercially available polyclonal anti-S and anti-s (Immucor, Norcross, GA) and single-source plasma or serum samples from donors or patients (human polyclonal anti-U). After the determination of the antigen profile, aliquots of 200 µL of 17 S–s–U– and 148 S–s+ samples were subjected to DNA analysis.

Genomic DNA Extraction

Genomic DNA was isolated by a whole-blood DNA extraction kit (Easy DNA, Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The DNA solutions were analyzed for quality by agarose gel electrophoresis. Quantification tests were not performed after extraction by this procedure; however, the expected yield was 3 to 12 µg of DNA.

Allele-Specific PCR

Allele-specific PCR (AS-PCR) for the S/s alleles were performed in all 17 S–s–U– and 148 S–s+ samples to characterize the alleles present. The sequences of primer combinations and control primers that amplified an unrelated gene (human growth hormone gene) were previously published. AS-PCR was carried out under the following conditions: 1× PCR buffer, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTP mix, 100 ng of sense and antisense primers, 100 ng of control primers, and 2.5 U Taq DNA polymerase. Two microliters of genomic DNA was used per 50-µL assay. Amplification was performed using a standard 35-cycle protocol, with an annealing temperature of 62°C.

GYPB Exon 5 Combination AS/PCR-RFLP Assay

Genomic DNA samples from the 4 nondeletion type S–s–U+var samples and 11 S–s+ samples were amplified with the GPB4/5, GPBV5S, and GPB5T primers, using a combination AS/PCR-RFLP assay to determine whether GYPB is present or absent and to distinguish the variant GYPB gene products in nondeletion type S–s– and S–s+ (GYPB*S silent gene) donors. The PCR products were digested with EcoRI (MBI, Fermentas, Amherst, NY) during an overnight incubation at 37°C. The uncut and digested products were analyzed on a 10% polyacrylamide gel.

Results

Serologic Analysis

RBCs from 165 blood samples were initially tested with polyclonal anti-S and anti-s by IAT-hemagglutination in gel test cards to determine the S/s profile. Of these, 17 samples were phenotyped as S–s– and 148 were S–s+. None of the S–s– samples reacted with our human polyclonal anti-U (n = 17) and were serologically classified as S–s–U–.

Allele-Specific PCR

Genomic DNA from the 165 samples was analyzed by AS-PCR assay for GYPB*S/s. The GYPB*S-specific primer pair amplified products in 4 of the 17 S–s– and 11 of the 148 S–s+ samples (Table 1). No amplification
was obtained in the other 13 S–s– samples, indicating that GYPB was deleted in these samples. The remaining 137 S–s+ samples only gave amplified products with the GYPB’s-specific primer, suggesting homozygosity or hemizygosity for GYPB (Fig. 1).

**Table 1.** Results of AS-PCR for GYPB*S/s

<table>
<thead>
<tr>
<th>Serologic results</th>
<th>Number</th>
<th>GYPB*S amplified product</th>
<th>No GYPB*S amplified product</th>
</tr>
</thead>
<tbody>
<tr>
<td>S–s–U–</td>
<td>17</td>
<td>4</td>
<td>13*</td>
</tr>
<tr>
<td>S–s+</td>
<td>148</td>
<td>11</td>
<td>137</td>
</tr>
</tbody>
</table>

*No GYPB*S or GYPB’s amplified products.

**Table 2.** Results of testing 17 S–s– DNA samples by AS/PCR-RFLP assay

<table>
<thead>
<tr>
<th>Number</th>
<th>Variant gene</th>
<th>Type of nucleotide change</th>
</tr>
</thead>
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<tr>
<td>2</td>
<td>GYP(P2)</td>
<td>+5 intron 5 (g&gt;t)</td>
</tr>
<tr>
<td>1</td>
<td>GYP(NY)</td>
<td>nt208 (G&gt;T) and nt230 (C&gt;T)</td>
</tr>
<tr>
<td>1</td>
<td>GYP(NY)/GYP(P2)</td>
<td>nt208 (G&gt;T) and nt230 (C&gt;T)/+5 intron 5 (g&gt;t)</td>
</tr>
<tr>
<td>13</td>
<td>GYPB deletion</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 3.** Results of testing 11 S–s+ DNA samples by AS/PCR-RFLP assay

<table>
<thead>
<tr>
<th>Number</th>
<th>Variant gene</th>
<th>Type of nucleotide change</th>
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</thead>
<tbody>
<tr>
<td>8</td>
<td>GYP(P2)</td>
<td>+5 intron 5 (g&gt;t)</td>
</tr>
<tr>
<td>3</td>
<td>GYP(NY)</td>
<td>nt208 (G&gt;T) and nt230 (C&gt;T)</td>
</tr>
</tbody>
</table>

The digested products from the four S–s– samples showed the following patterns: two were homozygous (or hemizygous) for the nucleotide change at +5 (g>t) of intron 5, consistent with variant GYP(P2), one was homozygous (or hemizygous) for nucleotide changes at nt208 (G>T) and nt230 (C>T), consistent with variant GYP(NY), and one was a heterozygous variant form for both GYP(NY)/GYP(P2) alleles.

In the 11 samples that phenotyped as S–s+, we found the following electrophoresis profile in the digested products: 8 had a nucleotide change at +5 (g>t) of intron 5, consistent with variant GYP(P2), and 3 presented with changes at nt208 and nt230, consistent with variant GYP(NY). The African Brazilians’ samples phenotyped as S–s+ were included in this study to extend the molecular characterization of variant GYPB. They provided evidence of a high prevalence (7.4%) of GYPB*S in this population. In samples that were apparently homozygous for the GYPB*s allele, it is not possible to determine whether the partner chromosome has a deleted GYPB.

**Discussion**

In the present study, the molecular background of variant forms of GYPB in Brazilian blood donors of African descent was determined for the first time through analyses of S–s– and S–s+ phenotypes. This is particularly important because the ability to detect these variant forms within a population of patients with sickle cell disease (SCD) or other hemoglobinopathies and of Black donors ensures the best match and transfusion safety. Moreover, the GYPB exon 5 combination assay is useful to readily identify GYPB-deleted donors for those patients whose antibody is compatible only with GYPB-deleted RBCs and also permits the identification of the silent S allele to resolve the discrepancies between hemagglutination and DNA-based assays. Clinical issues must be addressed for S–s–U– patients or for those who are S–s–U–*var*; as shown by Storry et al., they can produce anti-U when exposed to antigen-positive RBCs. This premise is consistent with other reports of clinically significant transfusion reactions in SCD patients and several cases of hemolytic disease of the fetus and newborn caused by anti-U. Transfusion of alloimmunized patients requiring S–s–U– RBC components is a challenge for any transfusion service because of the lack of well-characterized serologic screening reagents. PEG-IAT and MTS-gel with broadly reactive anti-U/GPB are the methods of choice to detect S–s–U–*var*. However, current knowledge of the molecular bases associated with expression of variant antigens makes DNA testing an important tool for both screening donors and typing patients. Therefore, it is now feasible to genotype donors to identify S–s–U– phenotypes.

In 1987, Huang et al. reported two unrelated individuals who exhibited the S–s–U– phenotype lacking GPB and observed that the absence of this protein correlated with deletion of GYPB. Gene deletion is a common mechanism for some human genetic disorders, most notably thalassemias.
Certain thalassemias occur in geographically defined groups of individuals, for which it has been presumed that the absence of the α or β globin gene may have represented a selective advantage. It is therefore of interest that the S–s–U– phenotype is prevalent among selected populations, in particular among Blacks from certain regions in Africa where the incidence of this phenotype ranges between 1 percent and 35 percent as compared with an incidence of less than 0.001 percent in the White population. However, several studies have associated the S–s– phenotype not only with the absence of the high-prevalence antigen, U, but also with weakened expression of U (the so-called U variant [U+var] phenotype). The S–s–U– phenotype is attributable to the deletion of GYPB, whereas the S–s–U+var phenotype is associated with a variant GPB protein that often expresses He.

Our findings were slightly different from previously reported data when comparing the molecular events implicated in the nature of GYPB variant forms found in our African Brazilian population. We report that 23.5 percent of African Brazilians phenotyped as S–s– presented a variant GYPB and that the +5 nucleotide change in intron 5 was the most common (50% of samples) mechanism for generating the S–s–U+var phenotype, followed by a change at nucleotide 208 and 230 (25% of samples with a variant GYPB) of exon 5 and a heterozygous change at nt208 and nt230 of exon 5 and +5 of intron 5 (25% of samples with a variant GYPB). However, in this study, we have not been able to define the zygosity status of the variant GYPB in the donors with S–s–U+var phenotype owing to the apparent inability of the AS/RFLP-PCR assay to detect the deleted GYPB gene when GYP(P2) or GYP(NY) is present as an apparent homozygote.
All samples from S–s– donors failed to react with human polyclonal anti-U. The absence of reactivity of our S–s–U+var samples with human polyclonal anti-U used in our laboratory raised questions about the nature of U on RBCs of normal and variant phenotypes. The molecular basis of U has not been identified so far; however, there is evidence to suggest a possible interaction of GPB with Rh-associated glycoprotein, RhAG, which could lead to conformational changes of variant GPB on the RBC surface. This is supported by the observation that some anti-U are compatible with all S–s– RBCs, even S–s–U+var. Apparently rare in an electronic literature search, the inclusion of African Brazilian blood samples phenotyped as S–s+ in this study, to complement and confirm the molecular mechanisms responsible for the S–s– phenotype, provided the knowledge of the high prevalence of GYPB*S in this population (7.4% of S–s+ samples), not described in the literature, showing heterozygous variant forms of GYP(P2) (73% of samples) followed by GYP(NY) (27% of samples). The implication of this molecular characteristic for blood transfusion practice is unknown and remains a challenge for future identification studies of variant forms; however, it is important that molecular methods incorporate the analysis of known GYPB variants, especially in a specific population.

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Rapid, single-subject genotyping to predict red blood cell antigen expression

S.L. SlezaK, S. Adams, H. Lee-Stroka, J.E. Martin, L. Caruccio, and D.F. Stroncek

Genotyping is useful to predict the expression of those RBC antigens for which antisera are difficult to obtain and to determine the probable phenotype of highly transfused patients, and it can be used to test stored DNA when a blood sample is not available. This study assessed a sequence-specific primer (SSP)-based genotyping system for blood group alleles suitable for the rapid testing of a small number of samples and assessed the use of stored whole blood. Genomic DNA was isolated from fresh and 1- and 2-week-old stored blood from 20 donors with known ABO and Rh phenotypes and was used for ABO, RHD, and RHCE genotyping using SSPs. The amplicons were analyzed using gel electrophoresis and a novel microfluidic on-chip electrophoresis system. Analysis of DNA from fresh and 1- and 2-week-old blood by SSP and gel electrophoresis yielded the correct ABO, RHD, and RHCE type in all samples, but with DNA from 2-week-old stored blood the amplicons were more difficult to visualize. Analysis of the same samples with the SSP on-chip electrophoresis assay correctly typed all samples except for one RHCE typing discrepancy of a fresh sample and one RHCE typing discrepancy of a 2-week-old sample. Analysis of amplicons by on-chip electrophoresis required one tenth the DNA that gel electrophoresis did and could be completed within 30 minutes compared with 2 hours with gel electrophoresis. Amplicons were also more readily visualized with on-chip electrophoresis. Fresh and 1- and 2-week-old samples could be ABO and RH genotyped with SSP. Analysis using on-chip electrophoresis was easier and more rapid than that using gel electrophoresis, but test reliability was slightly more variable.

As a result of the lack of available antisera for the typing of certain antigens and the need to perform higher resolution typing, molecular (DNA-based) assays have been developed to predict the expression of HLA, platelet, and neutrophil antigens.1–6 The molecular basis of almost all clinically significant RBC antigens is known, and methods for the analysis of blood group genotypes have been described.7–13 RBC genotyping is useful for predicting the phenotype of clinically important RBC antigens for which antisera are difficult to obtain. RBC genotyping is also useful for predicting the phenotypes of patients who are multiply transfused or whose RBCs are not available but their genomic DNA has been cryopreserved or otherwise saved.8–11,14

The method favored for genotyping varies among different types of laboratories. Large blood collection and processing centers require high-throughput systems to genotype large numbers of donors. Transfusion services and immunohematology reference laboratories are required to rapidly test one or a few patients or donors. Large blood centers will likely be using fresh blood samples for genotyping, whereas transfusion services or reference laboratories will be asked to test both fresh and stored blood samples.

The purpose of this study was to assess a genotyping system for blood groups that should be useful for laboratories that are required to rapidly type a few samples. A commercially available sequence-specific primer (SSP) method was used for ABO, RHD, and RHCE genotyping. To analyze the amplicons, a standard gel electrophoresis method was compared with a novel microfluidic on-chip electrophoresis method. Because on-chip electrophoresis allows for amplicon analysis within minutes rather than the hours required for gel electrophoresis, it may be a more convenient assay. As it is sometimes necessary to test patient samples collected and stored for several days, in addition to testing genomic DNA isolated from fresh blood samples, genomic DNA isolated from blood stored for 1 and 2 weeks at 2° to 6°C was also tested.

Materials and Methods

Study Design

Whole blood, 5 mL, from healthy subjects, was collected into EDTA and was used for ABO, RHD,
and RHCE genotyping and to determine the ABO and Rh phenotype. RBCs were serologically tested within 24 hours of sample collection. Genomic DNA was isolated from the blood samples within 48 hours of collection and from the same blood samples stored at 2° to 6°C for 1 and 2 weeks. The isolated DNA was amplified using SSP PCR, and the amplified DNA from each reaction was analyzed by both gel and on-chip electrophoresis. If samples generated unclear results, the SSP PCR and the electrophoresis were repeated.

**RBC Phenotyping**

RBCs were typed for ABO and D using gel microcolumns (Ortho-Clinical Diagnostics, Inc., Raritan, NJ) and for C, c, E, and e antigens using a tube agglutination method. The A2 phenotype was determined using an anti-A1 lectin by standard tube method (Immucor, Inc., Norcross, GA).

**DNA Isolation**

DNA was isolated from the whole blood samples using spin columns (QIAamp DNA Blood Mini Kit, QIAGEN, Valencia, CA) according to manufacturer’s instructions. After isolation, DNA concentration was determined by spectrophotometric analysis and the DNA was stored in QIAamp Elution Buffer at 2° to 6°C until use.

**ABO and RH Genotyping**

Genotyping for ABO (ABO-Type, BAGene, Biologische Analysensystem GmbH, Lich, Germany), RHD, and RHCE (RH-Type, BAGene) was performed using SSP via PCR methods. Approximately 100 ng/μL DNA was used for SSP amplification according to manufacturer’s instructions. The PCR conditions included an initial denaturation step for 5 minutes at 96°C, followed by 5 cycles of 10 seconds at 96°C, and 60 seconds at 70°C. The next 10 cycles were 10 seconds at 96°C, 50 seconds at 65°C, and 45 seconds at 72°C. The final 15 cycles were 10 seconds at 96°C, 50 seconds at 61°C, and 45 seconds at 72°C, and were followed by a final extension step of 5 minutes at 72°C.

The amplicons were analyzed by gel and on-chip electrophoresis. Standard gel electrophoresis was carried out on a 2% analytical gel prepared with SeaKem GTG agarose (BMA, Rockland, ME) and 1× TBE buffer (Cambrex Bio Sciences Rockland, Inc, Rockland, ME). After SSP PCR, the samples (9 μL) were electrophoresed at a constant 100 V for 75 minutes in a Gibco-BRL (Carlsbad, CA) 11.14 horizontal gel apparatus. The bromophenol blue dye front ran approximately 4 cm. To determine the size of the final amplicons 7 μL of Ready-to-Load 100bp Plus DNA ladder (Qiagen) was loaded in a separate lane.

On-chip electrophoresis was performed using lab-on-a-chip technology, which analyzes a very small volume of fluid in microfluidic channels etched in glass or plastic. After SSP PCR, 1 μL from each sample was loaded into one well of a 16-well chip (DNA chips, Agilent Technologies, Waldbronn Germany; Fig. 1). Only 12 of the 16 wells on the chip could be used for sample analysis. One of the remaining wells was loaded with 1 μL of DNA ladder, and three wells must be loaded with 9 μL of gel/dye mix (Agilent Technologies). The gel/dye mix is a sieving polymer matrix and fluorescent intercalating dye that is loaded into the appropriate wells on the chip and is forced through the microfluidic capillaries by applying pressure with a 1-mL syringe. With the capillaries filled, the chip acts as an electrical circuit, and the charged DNA sample can be separated by size as it is driven through the polymer matrix by a voltage gradient. The fluorescent dye is detected by laser-induced fluorescence. The chip was analyzed for 20 to 30 minutes with a bench-top chip reader (2100 Bioanalyzer, Agilent Technologies) linked to a computer, which produced a gel-like image (Fig. 1).
Results

Testing of Fresh Samples

Blood samples were tested from 20 donors (Table 1); 9 were group O, 7 A, 3 B, and 1 AB. RhD and RhCE phenotypes were also determined for the 20 donors; the sample group included 15 D+ and 5 D− samples (Table 1).

ABO genotyping

ABO genotyping of the 20 samples using genomic DNA isolated from fresh blood and analyzed by gel electrophoresis produced 2 samples that could not be interpreted. In one sample, all amplicons on the entire gel were poorly visualized, and in the other sample, the control amplicon in lane 2 was not detected. On-chip electrophoresis was able to provide better resolution of weak bands, but it was also more likely to detect additional bands, such as primer-primer dimers, that were not resolved in
the traditional gels (Fig. 2). When the amplicons from the same 20 donors were analyzed by on-chip electrophoresis, the results from 6 of the 20 subjects were difficult to interpret owing to problems with additional or smeared bands, or absent control amplicons in one or more lanes (Table 2). Repeat testing of these 8 samples yielded accurate ABO genotyping results in all 20 samples with both gel and on-chip electrophoresis.

**RHD and RHCE genotyping**

The DNA isolated from fresh blood was also used for RH genotyping assays, which yielded results similar to those produced by ABO genotyping. Of the 20 samples, 1 sample was difficult to interpret after standard gel electrophoresis and required repeat analysis because of a very weak control band in one lane. When the same 20 samples were analyzed by on-chip electrophoresis, 3 samples were of poor quality, and necessitated repeat analyses. An additional sample yielded an RHCE genotype, RHCE*Ce/Ce, that did not agree with the reported C+c+e+ Rh phenotype, and the RHCE*Ce/ce genotype obtained with gel electrophoresis. Repeat genotyping analysis of the sample with on-chip electrophoresis also yielded a genotype of RHCE*Ce/ce.

**Testing of Stored Samples**

**ABO genotyping**

On testing of the 1-week-old samples, the ABO genotyping assay yielded high-quality, accurate results, and repeat testing was required for only 1 of the 20 samples in both the gel and on-chip electrophoresis (Table 2). In general, the quality of both gel and on-chip electrophoresis analysis of DNA from 1-week-old samples was as good as or better than that obtained using DNA from fresh samples.

ABO genotyping results were more difficult to interpret using DNA isolated from 2-week-old blood samples, but generally the results of analysis with on-chip electrophoresis were better than with gel electrophoresis. ABO genotyping performed with DNA from blood stored for 2 weeks using gel electrophoresis resulted in five poor-quality samples owing to the absence of control amplicons, or both control and test amplicons. On-chip electrophoresis analysis resulted in difficult interpretation of three samples as a result of technical problems with a single lane. Generally, visualization of control and test amplicons with on-chip electrophoresis was superior to that with gel electrophoresis.

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Gel electrophoresis</th>
<th>On-chip electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABO</td>
<td>RHD/CE</td>
<td>ABO</td>
</tr>
<tr>
<td>Fresh blood</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1-week-old</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2-week-old</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

**RHD and RHCE genotyping**

RHD and RHCE genotyping could be performed with 1- and 2-week-old samples. The results of RH genotyping using DNA from 1-week-old samples were similar to those obtained using DNA from fresh blood. However, in some cases the results of RHD and RHCE genotyping using DNA from 2-week-old samples were more difficult to interpret, especially with on-chip electrophoresis.

When DNA from the stored samples was genotyped for RHD and RHCE using the gel electrophoresis system, four of the 1-week-old samples required repeat analyses, and none of the 2-week-old samples necessitated repetition. The 1-week-old samples required repeat analyses because of missing or weak control amplicons or both control and test amplicons in one or more lanes.

Of the eight stored samples that required additional RHD and RHCE genotyping with the on-chip electrophoresis system, two were 1 week old, and six were 2 weeks old. Seven of these samples required repeat testing as a result of weak or missing control or test amplicons, presence of extraneous bands, or poor amplicon alignment with the DNA ladder. In addition, one of the 2-week-old samples yielded a false-positive amplicon in a single lane that resulted in an incorrect RHCE genotype (RHCE*Cwe/ce) rather than the correct RHCE*Ce/ce genotype. However, on analysis using gel electrophoresis, as well as analysis of fresh and 1-week-old stored samples with both methods, the correct genotype was obtained. This suggests that the band responsible for the erroneous genotype result with the on-chip assay was an artifact not detected by traditional electrophoresis.

**Discussion**

We found that an SSP method could be used to determine ABO, RHD, and RHCE genotypes using...
leukocyte genomic DNA isolated from fresh whole blood and whole blood stored for 1 and 2 weeks at 2° to 6°C. Similar DNA yields were obtained after isolation at each of these times. Several samples had to be tested a second time because the initial results of either gel or on-chip electrophoresis were difficult to interpret. This was true for fresh and for 1- and 2-week-old samples.

The results of genotyping DNA isolated from 2-week-old blood were no less accurate, but they were more difficult to visualize than the results from fresh and 1-week-old blood. Amplicons from DNA isolated from 2-week-old blood tended to be weaker and, in general, more poorly visualized in gel electrophoresis than in on-chip electrophoresis.

Genotyping with SSP and gel electrophoresis was accurate for all whole blood samples that were fresh and 1 and 2 weeks old. Among the 120 ABO and RHD plus RHCE genotypes performed with on-chip electrophoresis, 2 were discrepant. Both involved RHCE genotyping, one using DNA from fresh blood, and the other using DNA from 2-week-old blood.

The on-chip electrophoresis technology is well suited for the rapid genotype analysis of a single patient. In comparison to gel electrophoresis, the on-chip method requires smaller sample size, can be carried out in minutes rather than hours, and is much easier to perform. However, the on-chip electrophoresis system used in this study is limited to the analysis of 12 amplicons at a time: enough amplicons to analyze ABO, but just short of the 13 amplicons required for RHD and RHCE analysis from a single subject. If both ABO and RHD/RHCE genotyping must be performed, the on-chip electrophoresis must be executed serially two times. Most gels used for electrophoresis also have a limited number of lanes, but multiple gels can be run simultaneously.

Although on-chip electrophoresis was able to better resolve amplicons, additional extraneous bands were more likely to be present than in gel electrophoresis. In addition, the on-chip electrophoresis was somewhat less technically reliable than gel electrophoresis. On-chip electrophoresis occasionally produced lanes that could not be analyzed as a result of amplicon absence or smearing, or the appearance of superfluous bands. As a result, when the PCR amplicon is of good quality, repeat electrophoresis is more likely required with on-chip electrophoresis than with gel electrophoresis. Fortunately, on-chip electrophoresis requires approximately one tenth the postamplification DNA that gel electrophoresis does, and if on-chip electrophoresis is used exclusively, enough DNA is produced by the SSP kits for multiple analyses.

The instrument for on-chip electrophoresis that was used in this study was not made for the analysis of clinical samples. However, a similar microfluidic capillary electrophoresis system designed specifically for the automated high-throughput analysis of clinical samples is now available (eGene HLA SSP, eGene Inc, Irvine, CA). This system will likely be more reliable then the one used in this study and results will likely be easier to interpret. The system contains modules that allow for optimal visualization of bands of a wide variety of specific sizes and intensities. In addition, the system is specifically designed for clinical testing rather than for use in the research laboratory, and we expect that the performance will be more robust. This system will automatically analyze 96 amplicons in less than 30 minutes and thus will allow the rapid analysis of multiple samples.

The analysis of the amplicons with on-chip electrophoresis was convenient, but a specialized instrument is required to analyze the microchips. We prepared our own agarose gels for electrophoresis; however, the time and labor involved with gel electrophoresis can be reduced by purchasing precast gels. Low-cost electrophoresis equipment is available for use with precast gels.

In addition to the SSP method used in this study, a sequence-specific oligonucleotide probe (SSOP) method that uses microparticles and slides is available for genotype determination of blood groups.\textsuperscript{15,19,20} In general, SSOP genotyping methods are more convenient for high-throughput testing; however, the SSOP method requires specialized equipment and staff training on equipment use. In addition, some high-throughput assays are only available in multiple test platforms, so reagents are wasted if only a single sample is tested.

The use of SSP methods like the one used in this study with on-chip or gel electrophoresis is well suited to the needs of a transfusion service, reference laboratory, or blood collection center that is required to genotype an occasional patient or donor. The SSP method uses equipment available in most laboratories: thermocyclers and electrophoresis equipment. The use of on-chip electrophoresis rather than agarose gel electrophoresis makes the SSP method especially fast and convenient for testing a limited number of samples.
In conclusion, ABO and RHD plus RHCE genotyping with SSPs was successful with genomic DNA isolated from fresh and 1-week-old blood samples. DNA from 2-week-old whole blood samples could also be used, but in some cases the results were difficult to visualize. Analysis using on-chip electrophoresis was easier and more rapid than gel electrophoresis, but test reliability was slightly more variable. ABO, RHD, and RHCE genotyping with SSPs is well suited for testing individual donors and patients, and is more convenient when used with on-chip electrophoresis.

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11. Rozman P, Dovc T, Gassner C. Differentiation of autologous ABO, RHD, RHCE, KEL, JK, and FY blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions. Transfusion 2000;40:936–42.


Anomalous expression of the Rh antigen, D, has occasionally been observed in patients with certain myeloproliferative disorders. Indeed, this phenomenon led to the tentative assignment of RH to the short arm of chromosome 1. PCR-based analyses were performed on DNA from an 82-year-old D+ Caucasian patient with chronic myeloid leukemia after her RBCs became D−. For nearly 7 years, the patient’s RBCs typed as strongly D+, but in March 2006, they typed weakly D+ and in August 2006 typed D− by both direct hemagglutination and the IAT. The D− typing persisted until the patient’s death in September 2006. To study the underlying cause of the change in D type, PCR-based assays were performed on DNA extracted from peripheral WBCs from the patient’s sample collected in August 2006. No amplification was obtained using primers designed to amplify RHD exons 5, 8, or 10, and intron 4. Very weak amplification was obtained using primers designed to amplify RHD exons 3, 4, or 7. Two assays that detect the hybrid Rhesus box showed deletion of RHD. Amplification of RHCE in the patient’s DNA was as efficient as that of control samples, and multiplex and PCR-RFLP assays predicted her RBCs would be C−E−c+e+. Based on finding a hybrid Rhesus box and absence of D-specific exons, we conclude that DNA from the patient’s WBCs carries a deleted RHD. This explains the molecular mechanism underlying the change from D+ to D−.

Key Words: blood groups, CML, Rh, DNA testing, molecular testing

Rh antigens are generally considered to be stable. Nonetheless, abnormal expression of some Rh antigens, notably D, has occasionally been observed in patients with certain myeloproliferative disorders, including chronic myeloid leukemia (CML). Although this phenomenon is usually associated with the presence of two RBC populations of different Rh phenotype in a patient’s peripheral blood, a few patients have a complete loss of certain Rh antigens. As early as 1974, this phenomenon led to the tentative assignment of RH to the short arm of chromosome 1.1 Later studies showed this to be true when the RH locus was assigned to chromosome 1p36.1.2,3 One D+ patient with myeloid metaplasia became D− owing to a chromosome translocation, and actually made anti-D plus anti-C.4 In another D+ patient with CML whose RBCs became D−, DNA analysis revealed a mutation within the RHD: a single nucleotide deletion (600Gdel) caused a frameshift that silenced RHD.5

To determine the underlying cause of the change in D type, we performed PCR-based analyses on DNA from a D+ patient with CML after her RBCs became D−. The patient had no evidence of a chromosome translocation and had not received a transplant. The results showed a remarkably precise deletion of RHD.

Case Study
The patient was an 82-year-old Caucasian female with CML. For nearly 7 years (May 1999 through February 2006), the patient’s RBCs typed as strongly D+ (3+). During these dates, no other Rh antigen typings were performed and O+ RBC components were transfused. In March 2006, the patient’s RBCs typed weakly D+ (1+) and she was transfused with two units of O+ packed RBC components. In August 2006, they typed D− with seven examples of reagent anti-D both by direct hemagglutination and by the IAT. At this time, her RBCs typed as C−E−c+e+. Repeat testing on the same sample and on a freshly collected sample confirmed the D− status of her RBCs. Furthermore, D− typings (by direct testing and IAT) were obtained on eight samples collected on different occasions between August and September 2006. From August 2006 until her death the patient was transfused with O− RBC components; she did not develop anti-D. Chromosome analysis was performed and showed no evidence of a chromosome translocation, and the patient had not received a stem cell transplant. To study the underlying cause of the change in D type, PCR-based assays were performed on DNA extracted from peripheral WBCs from the patient’s sample collected in August 2006. Unfortunately, the patient
died before we could obtain somatic cells (buccal epithelial cells, urine sediment, hair root, or tissue from an earlier biopsy) for extraction of DNA.

**Materials and Methods**

**Genomic DNA Extraction**

Genomic DNA was extracted using a DNA extraction kit (QIAamp DNA Blood Mini Kit, QIAGEN, Inc., Valencia, CA) from peripheral WBCs.

**PCR Amplification of RHD and RHCE**

*RHD* and *RHCE* were amplified using several primer pairs, which were synthesized by Life Technologies, Inc. (Gaithersburg, MD). Primer sequences, expected amplicon sizes, and annealing temperature are shown in Table 1. Five microliters of genomic DNA were amplified with 5 U of Taq DNA polymerase (HotStarTaq, QIAGEN, Inc.) in a 50-μL reaction mixture containing 1x PCR buffer, 0.2 mM dNTPs, 100 ng both primers, and specific magnesium chloride as listed in Table 1. PCR amplification was performed in a thermal cycler (Model 97000, Perkin Elmer, Norwalk, CT) under the following conditions: 35 cycles of 94°C for 20 seconds, 60°C, 62°C, or 64°C for 20 seconds, and 72°C for 20 seconds, followed by a final extension of 10 minutes at 72°C. PCR products were analyzed on a 1.2% agarose gel. A multiplex assay for *RHD* and *RHCE* was performed.

### Table 1. Primers used for PCR analyses

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Amplicon Size</th>
<th>Region</th>
<th>MgCl₂ and Annealing Temperature</th>
</tr>
</thead>
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<tr>
<td>RHDCEX3F</td>
<td>5’-TATTCGGCTGGCCACCATGA-3’</td>
<td>470 bp</td>
<td>RHD Exon 3</td>
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<td>RHD3S</td>
<td>5’-GGCTTGCCCGGGCAGG-3’</td>
<td>321 bp</td>
<td>RHD Exon 4</td>
<td>1.5 mM 64°C</td>
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<td>RHD4AS</td>
<td>5’-GCTTCAGACACCAGGGGAAC-3’</td>
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<td>RHD4</td>
<td>5’-TAAGCAGTTCACAGAGCAGG-3’</td>
<td>423 bp</td>
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<tr>
<td>HincD3R</td>
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<td>RHDARX7F</td>
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<td>262 bp</td>
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<tr>
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<td>5’-CATAGACATCCACACAGGGCA-3’</td>
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<td>CE4</td>
<td>5’-GGCAACAGGAGAGCTCCA-3’</td>
<td>474 bp</td>
<td>RHCE Exon 5</td>
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<td>CEX5</td>
<td>5’-CTGATCTTCTCTTGGGG-3’</td>
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<tr>
<td>Ex10F</td>
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<td>RHD3UT</td>
<td>5’-GTATTCCTACAGTGAATAAATGGTG-3’</td>
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<td>5’-GTGTCTGAGCGCTTCCATC-3’</td>
<td>115 bp</td>
<td>RHD</td>
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</tr>
<tr>
<td>RHI2</td>
<td>5’-GAAATCGTACACCCAGGC-3’</td>
<td>233 bp</td>
<td>RHCE</td>
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<tr>
<td>RHI3</td>
<td>5’-ATTAGCTGGCATGGTGTTG-3’</td>
<td></td>
<td>RHD and RHCE</td>
<td>1.5 mM 62°C</td>
</tr>
<tr>
<td>Rh-Hyb2-U</td>
<td>5’-ccacatcattttttctagag-3’</td>
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<td>RHD deletion: 1.5kb</td>
<td>2.0 mM 54°C</td>
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<tr>
<td>Rh-Hyb2-L</td>
<td>5’-aattatgcttctattacgg-3’</td>
<td></td>
<td>RHD upstream and downstream</td>
<td></td>
</tr>
<tr>
<td>HGH-F</td>
<td>5’-GCCTCCCCAACCATTCCC-3’</td>
<td></td>
<td>HGH: 429bp</td>
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<tr>
<td>HGH-R</td>
<td>5’-TACGGGATTTCTGTGTTT-3’</td>
<td></td>
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</tr>
<tr>
<td>Rh-Rez-7</td>
<td>5’-cctgtcccccatgtcattaccc-3’</td>
<td>3kb</td>
<td>RHD upstream and downstream</td>
<td>1.5 mM 58°C and 59°C</td>
</tr>
</tbody>
</table>
RHCE*E/e was analyzed by an allele-specific PCR-RFLP assay as described.7

Assays for the Presence of RHD Deletion

RHD zygosity was determined by PCR detection of the hybrid Rhesus box8 and by PCR-RFLP with Psfl.9 For the hybrid Rhesus box assay, PCR reactions were carried out in a total volume of 50 μL and contained 200 ng of genomic DNA, 1× PCR buffer, 2 mM MgCl2, 0.2 mM dNTPs, 10 μM of RH primers, and 2.5 μM of the human growth hormone (HGH) control primers, and 1 U of Taq DNA polymerase (QIAGEN, Inc). PCR amplification was performed in a thermal cycler (Model 2720, Perkin Elmer) under the following conditions: 30 cycles of 94°C for 30 seconds, 54°C for 45 seconds, and 72°C for 90 seconds, followed by a final extension of 5 minutes at 72°C. For the PCR-RFLP Psfl assay, PCR reactions were carried out in a final volume of 50 μL and contained 200 ng of genomic DNA, 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 15 μM each primer, and 2.5 U of HotStar Taq DNA polymerase (QIAGEN, Inc). PCR amplifications were performed under the following conditions: 10 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 120 seconds plus 5 seconds each cycle, followed by a final extension of 7 minutes at 72°C. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Results

Amplification of RHD and RHCE

No amplification was obtained using primers designed to amplify RHD exons 5, 8, and 10, and intron 4. Weak amplification was obtained using primers designed to amplify RHCE exons 3, 4, or 7. Repeat testing gave identical results. Using the same primers, in a D-positive control, RHD amplified as expected, and in a D-negative control did not amplify. Representative results are shown in Figure 1. Amplification of RHCE in the patient’s DNA was as efficient as that of control samples. Multiplex and PCR-RFLP assays predicted her RBCs would be C–E–c+e+ (not shown).

Assays for Rhesus Box

To further investigate the loss of D expression, an assay that detects the hybrid junction associated with the RHD deletion9 was performed. The assay was positive, indicating the patient had at least one RHD deletion. To further determine zygosity, a second assay was done9 to amplify a region termed the hybrid Rhesus box. Digestion of the products with the restriction enzyme Psfl gave a pattern consistent with samples homozygous for an RHD deletion (Fig. 2). These results confirm that the D-negative phenotype in this patient was associated with homozygous deletion of RHD.

To confirm the myeloid or somatic nature of this deletion would require analyses of DNA extracted from somatic cells. Unfortunately, the patient died before additional blood samples and a buccal smear as a source of somatic DNA could be obtained.

Discussion

At the time the patient’s RBCs typed D–, DNA extracted from her WBCs showed deletion of RHD and an apparently normal RHCE*ce. As the patient was Caucasian, she is more likely R0r than R0R0, and if so, the RHD was deleted from the chromosome carrying R0. This is the first report of DNA testing on a patient whose RBCs changed from D+ to D–, showing that the underlying cause is a specific deletion of
RHD. This mechanism is summarized in Figure 3. As we were unable to obtain DNA from somatic cells from this patient, we encourage others to study RHD and the hybrid Rhesus box in other such patients to determine whether deletion of RHD occurs specifically in hematopoietic cells. A recent paper by Kormoczi and coworkers\textsuperscript{10} describes a multi-technique approach to study nine patients with coexisting D+ and D– RBCs not attributable to transfusion, transplantation, or natural chimerism. In each case, their findings indicated myeloid-restricted loss of different stretches of the short arm of chromosome 1 (in one case the long arm of chromosome 1 was also involved), all of which encompassed the RHD/RHCE gene locus. Based on this study, loss of a part of chromosome 1, especially the short arm, is not as uncommon as previously believed.

In the case described here, the presence of the hybrid Rhesus box demonstrates the deletion of RHD, and the amplification obtained with some primer pairs shows the presence of low levels of RHD. Deletion of RHD but not RHCE demonstrates the precise nature of these events and provides insight into the role of the Rhesus boxes (which flank RHD but not RHCE) in the deletion of RHD in most D– Caucasians. This may provide an explanation as to why deletion of RHCE has not been observed.

**Acknowledgment**

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**References**


Ann Murdock, BS, and Deborah Assip, BS, Good Samaritan Hospital Medical Center, West Islip, NY; Kim Hue-Roye, Laboratory of Immunochemistry, New York Blood Center, New York, NY; Christine Lomas-Francis, MSc, and Zong Hu, BS, Laboratory of Immunohematology, New York Blood Center, Long Island City, NY; Sunitha Vege, MS, and Connie M. Westhoff, PhD, Molecular Blood Group and Platelet Testing Laboratory, American Red Cross, Penn-Jersey Region, Philadelphia, PA; and Marion E. Reid, PhD, (corresponding author) New York Blood Center, 310 East 67th Street, New York, NY 10065.
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.

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. 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 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 × 4 × 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
silent (synonymous), missense (nonsynonymous), or nonsense single nucleotide change and examples involving blood group antigens are illustrated.

**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.
allele results in a transferase with 21 amino acids more than in the A₁ transferase. 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.

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. 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
I identified the molecular bases associated with many antigens.\textsuperscript{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 \textit{Immunohematology}.

\section*{Acknowledgment}
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Marion E. Reid, PhD, Immunohematology Laboratory, New York Blood Center, 310 East 67th Street, New York, NY 10065.
Principles of PCR-based assays

K. Hue-Roye and S. Vege

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.

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

The molecular genetics world was revolutionized in 1983 with the advent of PCR, 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.

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).

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
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
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.

**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

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**Table 1. Characteristics of various PCR assays**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AS-PCR</th>
<th>PCR-RFLP</th>
<th>Multiplex</th>
<th>Sequencing</th>
<th>Real-Time PCR</th>
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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, Jsab/b, Kpa/b/c, Kmod-1), Kidd, Duffy (Fya/b/x, Fy null), MNS (M/N, S/s, U, Gp.Mur), Dombrock (Doa/b), Colton (Coa/b), and Diego (Diab/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 silicon 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, Kpa/b, Jsab/b, Jka/b, Fya/Fyb/ weakened Fyb/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 Fyb* (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.

**Acknowledgments**

We thank Robert Ratner for help in preparing the figures and Drs. Marion Reid and Connie Westhoff for their invaluable suggestions and comments.

**References**

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.

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\textsuperscript{var}, Fy\textsubscript{3}, Lu\textsuperscript{b}, D\textsuperscript{h}, Hy, Jo\textsuperscript{a}, Sc\textsuperscript{1}, LW\textsuperscript{a}, or Co\textsuperscript{b}. This can lead to improved patient care by having the appropriate antigen-negative blood available. 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\textsuperscript{a}/Fy\textsuperscript{b}, Jk\textsuperscript{a}/Jk\textsuperscript{b}, S/s, Do\textsuperscript{a}/Do\textsuperscript{b}, and Yt\textsuperscript{a}/Yt\textsuperscript{b}. 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\textsuperscript{a}, -Do\textsuperscript{b}, -Js\textsuperscript{a}, -Js\textsuperscript{b}, -C\textsuperscript{w}, -V, and -VS. These assays are particularly valuable in the
Dombrock blood group system; typing for Do\(^+\), Do\(^-\), Hy, and Jo\(^+\) 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\(\sim\), E\(\sim\), S\(\sim\), K\(\sim\), Fy(a\(\sim\)), and Jk(b\(\sim\)), 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\(^a\) 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 Sc\(\sim\) donors, as well as three c\(\sim\) and many c\(\sim\), and s\(\sim\) donors that have been confirmed by serology.
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, Fya/Fyb, and Jka/Jkb. 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.
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.

**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
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, Fya, Fyb, Jka, Jkb, S, s) for “common” combinations and more than 200 donor samples for 15 high-prevalence antigens (U, hrA, hrB, Rh17, k, Kp, Js, Yt, Gy, Hy, Jo, Ge, PP, Pk, Vel, Lan). By PCR-based assays, we screen approximately 10 samples by LDT for V, VS, hrA, hrB, Do, Do, Hy, and Jo, 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.

**Acknowledgments**

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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.


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. 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. 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 RH D pseudogene that, when present, prevents expression of D on the RBC surface. 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.

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, 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 BanI, is shown in Figure 1. An
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 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

<table>
<thead>
<tr>
<th>Predict the RBC phenotype when</th>
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<tr>
<td>• A patient was recently transfused</td>
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<tr>
<td>• 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</td>
</tr>
<tr>
<td>• A fetus is at risk for HDFN</td>
</tr>
<tr>
<td>• Antibody is weak or not available, e.g., anti-Do(^a), anti-Js(^a), anti-V (^b)</td>
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<table>
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<tr>
<th>Determine zygosity, particularly RHD</th>
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<tr>
<td>Resolve discrepancies, e.g., A, B, D, C, c, e</td>
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<th>Distinguish allo- from autoantibodies</th>
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<tr>
<td>Detect weakly expressed antigens (e.g., Fy(^b) with the Fy(^a) phenotype); when patient is unlikely to make antibodies to transfused antigen-positive RBCs</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+C+E–c+e+</td>
<td>Predominantly used for patients with anti-E and anti-K.</td>
</tr>
<tr>
<td>K–k+</td>
<td>Predominantly used for patients with anti-K.</td>
</tr>
<tr>
<td>Jk(a+b–), S+s+, Fy(a+b+)</td>
<td>Predominantly used for patients with anti-Jk(a) and anti-Jk(b).</td>
</tr>
</tbody>
</table>

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 absorptions with R,R0, R,R0, 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/Cc, RHCE/c,c, 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+GcE–c+c+, K–k+, Jk(a+b–), 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.

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*, Do, Hy, Jo), 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+b+w+b+), 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 Ks, 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).

Elute 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 Ks 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 NalIII was used for PCR-RFLP analysis of KEL*3/KEL*4 (Kp*/Kp) 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.

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 M0M8, GP.Hi/GP.Hi (Mi.V/Mi.V), and GP.JL/M0 (Mi.XI/M0) 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 glycoporphin 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+) when tested with eight examples of monoclonal anti-Wr b. This altered expression of Wr b was consistent with the possibility...
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.

\textbf{GYPA} encodes GPA and exon 4 of \textbf{GYPA} encodes
the portion of GPA closest to the RBC membrane; therefore, DNA sequencing of exon 4 was per-
formed. 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 anti-
gen 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 242T>G change ablates a DNA cleavage
site for the restriction enzyme \textit{RsaI}. 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.

\textbf{AS A TOOL TO IDENTIFY ANTIBODIES DETECTING CROMER
BLOOD GROUP SYSTEM ANTIGENS}

The Cromer blood group system currently con-
tains 12 antigens of high prevalence and 3 antigens
of low prevalence; they are carried on the comple-
ment 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 as-
says. 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.\textsuperscript{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 anti-
body 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 find-
ing 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 ana-
lyzed. Regions of DNA that included exons 2 to 6

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{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.}
\end{figure}
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.

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

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

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 hrS. Several genotypes have been associated with the hrS 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 FyX phenotype (FY*A/FY*X) and a greatly reduced expression of the Fy antigen, caused by a change at nucleotide 265, at the FY*B locus, is unlikely to make anti-Fy 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 antigen attributable to the FyX phenotype is difficult to detect serologically with most commercial anti-Fy reagents. Occasionally, a reagent that detects the Fy antigen 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

**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, Rhnull, Knull, 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

The authors would like to thank Robert Ratner for his assistance in preparation of the manuscript and the figures.

References

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The potential of blood group genotyping for transfusion medicine practice

C. M. WESTHOFF

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.
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

<table>
<thead>
<tr>
<th>Category</th>
<th>Antigen target for extended match</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient with sickle cell disease (prophylactic transfusion)</td>
<td>C, E, K altered C, partial D, partial e Fy(^a), Jk(^a), Jk(^b), S, s</td>
</tr>
<tr>
<td>Patient with warm autoantibodies</td>
<td>C, c, E, c, K Jk(^a), Jk(^b), Fy(^a), Fy(^b), S, s</td>
</tr>
<tr>
<td>Patient with alloantibody(ies)</td>
<td>C, c, E, c, K Jk(^a), Jk(^b), Fy(^a), Fy(^b), S, s</td>
</tr>
<tr>
<td>Women of childbearing age and female children</td>
<td>D, K, c</td>
</tr>
</tbody>
</table>
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 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–, Uvar, and HY– Jo(a–) samples, but does not target any of the Jk(a–b–) i.e., JKnull or Gregory (Gyα–) 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


15. Ness PM. To match or not to match: the question for chronically transfused patients with sickle cell anemia. Transfusion 1994;34:558–60.


Connie M. Westhoff, SBB, PhD; Scientific Director; Molecular Blood Group and Platelet Testing Laboratory, American Red Cross, Penn-Jersey Region, Philadelphia, PA 19130.
Letter From the Editors

To Contributors to the 2008 Issues

The journal depends on readers, authors, editorial board, peer reviewers, and our Penn-Jersey staff. We wish we could thank all of you personally, but doing so is not practical. Instead, we thank each of you as members of an honored group.

First and foremost, we thank the authors for their reviews, scientific articles, case reports, book reviews, and letters to the editors that come not only from the United States but from many countries of the world. This has given the journal an international flavor.

Our editorial board is a prestigious one and we depend on them, not only for peer reviews, but for guidance in policy and suggestions for improvements. Special thanks go to our medical editors, who review every article for medical content, and to our technical editors, who read every article for technical content. The current board is listed by name in the front of each issue of the journal.

Our peer reviewers did a wonderful job in 2008. In each December issue we list them by name with thanks to each.

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We also want to thank the office staff at Penn-Jersey, Marge Manigly and Judy Abrams, for their help in preparing the journal for press. They manage the manuscript submissions, keep up with subscriptions, and many other behind-the-scenes tasks. We also thank Mary Tod, our copy editor, Lucy Oppenheim, our proofreader; and Paul Duquette, our electronic publisher.

Finally, thanks go to our readers, whose enthusiasm and interest in the journal make it all worthwhile.

Sandra Nance
Connie M. Westhoff
EDITORS-IN-CHIEF

Cindy Flickinger
MANAGING EDITOR
Neonatal and infant platelet transfusions
A reader has informed the editors of *Immunohematology* that there is an error on page 16, second column, second paragraph, first sentence. The sentence should read “The standard dose for neonates is based on body weight and is the same for either apheresis platelets or whole blood–derived platelets at 5 to 10 mL/kg for an increase of 50,000/μL in the platelet count.”
Meetings!

**June 4–5  Blood Bank Association of New York State (BBANYS)**

The 58th annual meeting of the Blood Bank Association of New York State (BBANYS) will be held June 4 and 5, 2009, in New York, New York. An AABB new assessor training session will be held on June 3, 2009. Topics to be presented at the meeting include these:

- Scientific Sessions
- Pathogen Reduction/Inactivation/Additives
- Thrombopoietin and Platelet Transfusions
- Mesenchymal Precursor Cell Therapy
- Cord Blood Transplant for Sickle Cell Disease
- Biovigilance
- Age of Red Blood Cells at Transplant
- Battlefield Medicine
- TRALI/TACO
- Technical/Immunohematology Serologic Sessions
- Damp Workshop
- Antibody Case Studies
- Management and Nursing Sessions
- Managing for Excellence
- Patient Safety
- Extracorporeal Photopheresis
- Transfusion Reactions
- Regulatory Update

For more information, contact the Web site at http://www.bbanys.org
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Are you working in NHS or National Blood Service and looking for training?
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Applications are invited from medical or science graduates to study for the MSc in Transfusion and Transplantation Sciences. The course is run jointly by The Department of Cellular & Molecular Medicine, University of Bristol and the Bristol Institute of Transfusion Sciences.

The course starts in October 2009 and can be studied full-time for 1 year or **part-time over 2 or 3 years by block release**.

The course aims to develop your interest, knowledge and understanding of the theory, practical techniques and research relating to the science of transplantation and transfusion medicine.

For example,

- How is blood processed?
- When do we give platelet transfusions?
- How is tissue engineering being used to replace heart valves?
- What causes haemolytic anaemia?
- How do we reduce the transfusion related risk of HIV and vCJD?

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The lecture units are: Haemopoiesis, Immunology, Platelets and coagulation, Blood groups, Haematological diseases, Blood donation, Blood components, Clinical transfusion, Transfusion transmitted infections, Stem cell transplantation, Solid organ transplantation and Tissue engineering.

**The course is accredited by The Institute of Biomedical Sciences and directed by Professor David Anstee and Dr Tricia Denning-Kendall.**

For further details visit:

[http://www.blood.co.uk/ibgrl/MSc/MScHome.htm](http://www.blood.co.uk/ibgrl/MSc/MScHome.htm)

or contact:

Dr Tricia Denning-Kendall,
University of Bristol, Geoffrey Tovey Suite,
tel 0117 9912093, e-mail P.A.Denning-Kendall@bristol.ac.uk
Blood Group Antigens & Antibodies

A guide to clinical relevance & technical tips

BY

MARION E. REID AND CHRISTINE LOMAS-FRANCIS

The authors are using royalties generated from the sale of this pocketbook for educational purposes to mentor people in the joys of immunohematology as a career. They will accomplish this in the following ways:

• Sponsor workshops, seminars, and lectures
• Sponsor students to attend a meeting
• Provide copies of the pocketbook

(See www.sbbpocketbook.com)

The book, which costs $25, can be ordered in two ways:

1. Order online from the publisher at: www.sbbpocketbook.com

2. Order from the authors, who will sign the book. Send a check, made payable to “New York Blood Center” and indicate “Pocketbook” on the memo line, to:

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   New York, NY 10021

   Please include the recipient’s complete mailing address.

About the book

This compact “pocketbook” from the authors of the Blood Group Antigen FactsBook is a must for anyone who is involved in the laboratory or bedside care of patients with blood group alloantibodies. The book contains clinical and technical information about the nearly 300 ISBT recognized blood group antigens and their corresponding antibodies. The information is listed in alphabetical order for ease of finding—even in the middle of the night. Included in the book is information relating to:

• Clinical significance of antibodies in transfusions and HDN.
• Number of compatible donors that would be expected to be found in testing 100 donors. Variations in different ethnic groups are given.
• Characteristics of the antibodies and optimal technique(s) for their detection.
• Technical tips to aid their identification.
• Whether the antibody has been found as an autoantibody.
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- Molecular analysis for HPA-1a/1b

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I. GENERAL INSTRUCTIONS

Before submitting a manuscript, consult current issues of Immunohematology for style. Double-space throughout the manuscript. Number the pages consecutively in the upper right-hand corner, beginning with the title page.

II. SCIENTIFIC ARTICLE, REVIEW, OR CASE REPORT WITH LITERATURE REVIEW

A. Each component of the manuscript must start on a new page in the following order:
   1. Title page
   2. Abstract
   3. Text
   4. Acknowledgments
   5. References
   6. Author information
   7. Tables
   8. Figures

B. Preparation of manuscript

1. Title page
   a. Full title of manuscript with only first letter of first word capitalized (bold title)
   b. Initials and last name of each author (no degrees; all CAPS), e.g., M.T. JONES, J.H. BROWN, AND S.R. SMITH
   c. Running title of ≤40 characters, including spaces
   d. Three to ten key words

2. Abstract
   a. One paragraph, no longer than 300 words
   b. Purpose, methods, findings, and conclusion of study

3. Key words
   a. List under abstract

4. Text (serial pages): Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Survey results and review papers may need individualized sections
   a. Introduction
      Purpose and rationale for study, including pertinent background references
   b. Case Report (if indicated by study)
      Clinical and/or hematologic data and background serology/molecular determinations
   c. Materials and Methods
      Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient’s names or hospital numbers.
   d. Results
      Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
   e. Discussion
      Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction

5. Acknowledgments: Acknowledge those who have made substantial contributions to the study, including secretarial assistance; list any grants.

6. References
   a. In text, use superscript, Arabic numbers.
   b. Number references consecutively in the order they occur in the text.

7. Tables
   a. Head each with a brief title; capitalize the first letter of first word (e.g., Table 1. Results of . . .) use no punctuation at the end of the title.

b. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.

8. Figures
   a. Figures can be submitted either by e-mail or as photographs (5 × 7 glossy).
   b. Place caption for a figure on a separate page (e.g., Fig. 1 Results of . . .), ending with a period. If figure is submitted as a glossy, place first author’s name and figure number on back of each glossy submitted.
   c. When plotting points on a figure, use the following symbols if possible:
      ○ ● ▲ ▼ □ ▬ ■

9. Author information
   a. List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable.

III. EDUCATIONAL FORUM

A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:
   1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
   2. Annotated conference proceedings

B. Preparation of manuscript

1. Title page
   a. Capitalize first word of title.
   b. Initials and last name of each author (no degrees; all CAPS)

2. Text
   a. Case should be written as progressive disclosure and may include the following headings, as appropriate
      i. Clinical Case Presentation: Clinical information and differential diagnosis
      ii. Immunohematologic Evaluation and Results: Serology and molecular testing
      iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
      iv. Recommended Therapy: Include both transfusion and nontransfusion-based therapies
      v. Discussion: Brief review of literature with unique features of this case
      vi. Reference: Limited to those directly pertinent
      vii. Author information (see II.B.9.)
      viii. Tables (see II.B.7.)

IV. LETTER TO THE EDITOR

A. Preparation

1. Heading (To the Editor)
2. Title (first word capitalized)
3. Text (written in letter [paragraph] format)
4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
5. References (limited to ten)
6. Table or figure (limited to one)

Send all manuscripts by e-mail to immuno@usa.redcross.org
Becoming a Specialist in Blood Banking (SBB)

What is a certified Specialist in Blood Banking (SBB)?
- Someone with educational and work experience qualifications who successfully passes the American Society for Clinical Pathology (ASCP) board of registry (BOR) examination for the Specialist in Blood Banking.
- This person will have advanced knowledge, skills, and abilities in the field of transfusion medicine and blood banking.

Individuals who have an SBB certification serve in many areas of transfusion medicine:
- Serve as regulatory, technical, procedural, and research advisors
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- Develop, validate, implement, and perform laboratory procedures
- Analyze quality issues, prepare and implementing corrective actions to prevent and document issues
- Design and present educational programs
- Provide technical and scientific training in blood transfusion medicine
- Conduct research in transfusion medicine

Who are SBBs?
Supervisors of Transfusion Services
Managers of Blood Centers
LIS Coordinators
Educators
Supervisors of Reference Laboratories
Research Scientists
Consumer Safety Officers
Quality Assurance Officers
Technical Representatives
Reference Lab Specialists

Why be an SBB?
Professional growth  Job placement  Job satisfaction  Career advancement

How does one become an SBB?
- Attend a CAAHEP-accredited Specialist in Blood Bank Technology Program OR
- Sit for the examination based on criteria established by ASCP for education and experience

Fact #1: In recent years, the average SBB exam pass rate is only 38%.
Fact #2: In recent years, greater than 73% of people who graduate from CAAHEP-accredited programs pass the SBB exam.

Conclusion:
The BEST route for obtaining an SBB certification is to attend a CAAHEP-accredited Specialist in Blood Bank Technology Program

Contact the following programs for more information:

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