The BloodGen project: toward mass-scale comprehensive genotyping of blood donors in the European Union and beyond


INTRODUCTION

Human blood group antigen expression is governed by polymorphic variation in protein and carbohydrate structures that extend from the surface of the erythrocyte membrane. Carbohydrate-dependent antigens (in ABO, P1, H, LE, GLOB, and I systems) are variations in polysaccharide antennae attached to glycoprotein or glycolipid molecules. The genetic bases of carbohydrate-dependent blood groups are determined by variation in glycosyltransferase sequences expressed by erythroid (and other) cells, which affect the enzyme specificity or efficacy during polysaccharide synthesis within the Golgi compartment.1 Protein-dependent antigens (in RH; KEL; FY; JK; GIL; CO; DO; MNS; CROM; DI; IN; CH/RG; KN; GE; LW; LU; OK; SC; YT; XG; JMH; RAPH; and XK systems) are caused predominantly by single-nucleotide polymorphisms (SNPs) in the structural genes encoding erythrocyte membrane proteins.2 These proteins have diverse functions, such as transporters and channels (RH, DI, CO, GIL, JK, XK); structural proteins (GE, MNS); complement control proteins (CH/RG; CROM; KN); adhesion and signaling molecules (LW, LU, OK, SC, XG, IN, RAPH, JMH); chemokine receptor (FY), and membrane-bound enzymes (KEL, DO, YT). The CH/RG blood-group-active molecules are the C4 component of complement and are adsorbed from plasma onto the surface of red blood cells (RBCs) (for reviews, see the articles of Avent3 and Daniels4). The determination of the molecular bases of blood group antigens has been intensely researched, with most clinically significant alleles defined. Most blood group variation is the result of SNPs in the corresponding genes, e.g., K/k;5 Fya/Fyb6 and Rh C/c and E/e.7,8 However, SNPs are not the sole genetic mechanism for blood group polymorphism. In the ABO system, hybrid alleles due to recombination or gene conversion events can lead to unexpected phenotypes and erroneous genotyping results.9 In D– phenotypes, the RHD gene is deleted,10 or an RHD pseudogene (RHDγ)11 or hybrid RHD-RHCE genes can be present.12 Other RHD alleles include partial D phenotypes, which may induce anti-D when exposed to D+ blood. Partial D phenotypes can be caused by SNPs in the RH gene that alter exofacial amino acids, e.g., DNU Gly353Arg13 and DVII Leu110Pro.14 Others are caused by hybrid RHD-RHCE-RHD genes, probably created by gene conversion, e.g., DVI is a hybrid RH gene composed of the following gene structures: type 1: RHD-RHCE(4-5)-RHD;15,16 type 2: RHD-RHCE(4-6)-RHD;16,17 type 3: RHD-RHCE(3-6)-RHD;18 and type 4 RHD-RHCE(2-5)-RHD.19 Weak D phenotypes are caused by
SNPs in the RHD gene which alter amino acids that are important for Rh protein subunit assembly,20,21 as are Del (DEL) phenotypes which are often caused by mutations at consensus splice-site junctions22 in the RHD gene. In DEL phenotypes, there is a massive reduction in D antigen expression. The genetic basis of the Fy(a–b–) phenotype of people of African descent is unlike all other blood group polymorphisms and is caused by a promoter region mutation that disrupts a GATA-1 binding site.23 This mutation abolishes the erythroid expression of the Fy glycoprotein, whereas it is expressed normally in nonerythroid tissues in the same individual.

We describe in this article the development and demonstration of a DNA array-based approach for the definition of blood group status using blood from donors or patients as starting material. We suggest that this approach, especially when applied to the Rh system, is more accurate than serology and may be applied as a replacement technology for blood grouping.

CURRENT BLOOD GROUP GENOTYPING

Blood grouping by deoxyribonucleic acid (DNA)-based techniques is currently confined to situations where blood cells cannot be typed or are dangerous to obtain.24-26 This is predominant in the prenatal definition of RHD and other blood group polymorphisms where fetuses are at risk of hemolytic disease of the fetus and newborn.27,28 Additionally, multiply transfused patients, whose phenotype is difficult to define serologically because of the presence of donor RBCs in the peripheral blood, is another situation where molecular genotyping is the preferred methodology. In the presence of a positive direct antiglobulin test, because of autoantibodies, serologic typing can be impaired.

In the future, genotyping of blood group is likely to gain much broader application. Significant technical advances have been achieved in the implementation of high-throughput techniques in molecular genotyping, especially with respect to germ-line variation in the human genome sequence.29,30 It is likely that mass-scale genotyping technologies will be commonplace for population genetics, e.g., neonatal screening for inborn errors of metabolism such as cystic fibrosis.31 When such technologies are the norm, it makes perfect sense for every individual to have his or her blood group and human leukocyte antigen genotype defined after birth. This information, of high relevance to that individual, will follow him or her during life. Drawbacks in the use of genotyping to predict a phenotype are false-positive and false-negative results due to undescribed mutations. Although exceptional, it should also be realized that twin-to-twin blood exchange in fetal life or hematologic malignancies can cause blood group chimerism of which at least one has been defined at the molecular level26 and thus somatic mutations can cause alteration in blood group phenotypes. In addition, infections, cancer, and pregnancy are some of the conditions known to have the capacity to change an individual’s blood group, either through well-defined mechanisms such as bacterial enzymes altering the glycalyx of the cells, through temporary down regulation of mRNA levels in cancer, or by unknown routes.

BLOOD GROUP GENOTYPING AND THE EUROPEAN COMMUNITY (EC) BLOOD DIRECTIVE

Initial genetic characterization of each of the blood group systems began in 1986 with MNS,33 followed in 1990 with ABO,34 and RH.35,36 The molecular basis of most of the clinically significant blood group antigens has now been defined. This intense activity now provides an opportunity to implement high-throughput genotyping to define blood group genotype, and may soon provide a viable alternative to blood group serology in certain aspects of routine blood banking. Throughout the European Union (EU), routine blood group serology is focused on the detection of the major clinically significant blood groups, ABO, RH, KEL, FY, JK, and MNS, and is reliant on the efficient detection of blood-group-specific antibodies in patients’ sera using the pretransfusion screening with antibody detection RBCs or crossmatching test before transfusion. In addition, serologic screening efforts to find blood donors with rare blood group phenotypes for alloimmunized patients are carried out when appropriate reagents are available. Despite the inherent safety of current blood transfusion practice, primary alloimmunization caused by the currently accepted presence of blood group incompatibility between the donor and the recipient can cause severe (acute or delayed) transfusion reactions resulting in prolonged hospitalization or even death, and is still very much an issue of transfusion safety.37 In the EU, before the blood directive of 2002 (2002/98/EC),38 two hemovigilance systems were in place, the hemovigilance system in France, which is mandatory, and the voluntary Serious Hazards of Transfusion scheme in the UK. The major difference between the two reporting systems is that the French mandatory system indicated 300 transfusion-related events in 100,000 blood products, whereas the British system revealed only 10 incidents in the same number of products. Hemovigilance is a requirement for all blood services within the EU as indicated in recital 18 of the EC Blood directive. The directive also states in recital 9 that “...the European Parliament stressed the importance of ensuring the highest level of blood safety and has reiterated its continued support for the objective of Community self-sufficiency.” The occurrence of delayed hemolytic transfusion reactions (DHTRs), e.g., those caused by anti-Jk*, at present cannot...
be effectively eliminated by serologic typing, by screening, and by crossmatching; thus JK genotyping of patients and donors and subsequent preventive matching would eliminate the occurrence of delayed hemolytic transfusion reactions due to anti-Jkα. It is our opinion that mass-scale genotyping of all blood donors and patients within the EU would lead to a marked reduction in alloimmunization events and would improve the transfusion outcomes for vulnerable patients, e.g., multiply transfused individuals and women of childbearing age.

STRUCTURE AND OBJECTIVE OF THE BloodGen CONSORTIUM

BloodGen (http://www.bloodgen.com) was a concept conceived in response to the development of array technology when applied to molecular blood grouping. Many consortium members have been involved in the definition of the molecular basis of blood groups since 1990.1,8,9,11,18-20,39-48 Based on this collective knowledge, a consortium was assembled and a submission to the final call of Framework V of the EC’s Quality of Life and Management of Living Resources program was made. The project was submitted as a Research and Technical Development demonstration project, with the intention of demonstrating the commercial viability of genotyping using glass arrays of oligonucleotides, and the utilization of a fluoro allele-specific primer (single-sequence primer). Progenika Biopharma SA (Derio, Spain) and Biotest (Dreieich, Germany) led the development of such assays for blood group genotyping using proven technical platforms. The consortium included blood banks (Barcelona and Prague) and academic research institutes and foundations (University of the West of England, Bristol, Sanquin, Bristol Institute for Transfusion Sciences, Ulm, and Lund). The majority of the resources of the project were directed toward the technical and academic support of the production of the Bloodchip product. Bloodchip will be Conformité Européenne (CE)-marked following the completion of the project and will be launched as a commercial product following dissemination events by Progenika Biopharma SA and by Sanquin.

TECHNICAL ASPECTS

The first task of the project was to select a list of SNPs, deletions, and insertions corresponding to blood group alleles to incorporate onto the Bloodchip testing platform. Initially 94 SNPs were selected, but this was expanded to 106 during the project to include newly discovered alleles and alleles of the human platelet antigen systems. To detect each allele, multiplex (MPX) PCRs were designed to amplify all regions of blood-group-specific genes encoding the causative alleles. Initially, three MPXs were designed: ABO, RHD, and MPX amplifying regions of GYPB and GYPB (MNS)—RHCE, DO, KEL, CO, JK, DI, and FY genes. Designs were incorporated into the various MPXs to ensure RHD and RHCE specificity. We found that the optimal PCR amplification conditions were such that ABO and RHD MPX PCRs could be combined. The specificity and high yields of each MPX product were achieved by incorporating within the PCR mixes primers with Multiplex Amplifiable Probe Hybridization (MAPH) tags to the 5’ end of each primer as described by Beiboer et al.49 The PCR probe mixes contained both MAPH-tagged and non-tagged blood-group-specific primers, which in combination were capable of amplifying nucleotide sequences encompassing all SNPs required for detection on Bloodchip (see Fig. 1).

While the MPXs were being developed by the consortium, a panel of rare blood group genomic DNAs was assembled as a biobank, and used by the consortium to validate the MPXs and by Progenika Biopharma SA to develop probes that are covalently bound to Bloodchip and designed to hybridize to each target SNP. Bloodchip uses similar technical aspects to Lipochip, which is used to detect familial hypercholesterolemia by Progenika Biopharma SA.50 Briefly, the MPX PCR products are labeled with Cy dyes and fragmented by DNAse I before hybridization to the chip using stringent conditions (see Fig. 2). The allele-scoring process is then achieved following the scanning of Bloodchip with an array scanner, then using bespoke software (Progenika) to score the different blood group alleles. This was achieved by the application of an algorithm to the data, which defines clusters of fluorescence intensity to homozygosity for a particular allele. Scoring is only possible when the fluorescence signals are above a certain threshold (are scored in rank), and hybrid alleles that cause, e.g., partial D phenotypes, are scored when certain signals are out of rank. For example, a DVI allele would be scored if RHD exons 4 and 5 signals were out of rank, but all others were detected normally.

CLINICAL TRIALS

The efficacy of Bloodchip has been determined by a series of small-scale clinical trials, conducted in the UK, Spain, Germany, Czech Republic, and Sweden, initially using a cohort of archived genomic DNAs extracted from individuals with very rare blood groups. This exercise was repeated several times to optimize the design of both MPX PCRs and probe sequences on Bloodchip. Members of the consortium are currently engaged in a large clinical trial where Bloodchip is being used on a panel of 3000 ABO, Rhesus CcDeE, and K-type individuals for Conformité Européenne (CE) marking purposes, as laid out in the common technical specification for diagnostic reagents of blood group specificity. The goal is to implement the technology in selected EU countries to target at-risk patient
groups and a relevant fraction of blood donors where blood group genotyping will have the most impact.

**THE FUTURE OF BLOOD GROUP GENOTYPING**

It has been argued that high-throughput genotyping methodology (with some caveats) could replace blood group serology for donors, especially considering the mass-scale virus screening programs in place which may eventually be performed on individual donations. Although viral nucleic acid testing schemes require different technical manipulations (such as reverse transcription), they already meet the requirements of industrial-scale manipulation of large numbers of donor and eventually patient samples. This could readily be extended to patients’ samples in large diagnostic testing laboratories. It could be argued that blood group genotyping should be implemented for all blood donors, especially those who donate repeatedly, to obtain a fully typed inventory, facilitating the direct availability of antigen-negative blood for alloimmunized recipients. However, to be truly effective, recipients must also be typed to allow “preventive matching,” which will imply clinically relevant blood group mismatches to be fully scrutinized. Genotyping all donors would allow the identification of “high risk” blood group alleles, e.g., Jk⁺, Fy⁺, and S, within the donor cohort. Some studies have included the mass-scale genotyping of RhD− blood group units as a quality control exercise to remove weak D, partial D, and DEL units that have caused alloimmunization to the D antigen. This approach may be valid, especially considering the fact that some “D−” mothers, when retested by RHD genotyping methodology were in fact revealed to be normal RhD+ individuals who had been mistyped either by serologic or by clerical errors.

---

**Fig. 1.** MAPH MPX PCRs used to amplify blood-group-specific genes. This figure illustrates the PCR amplicons from the two Blood-chip MPX PCRs: (A) ABO and RHBD MPX PCR (15 fragments) and (B) MPX PCR for GYPH and GYPA (20 fragments). RHCE, DO, KEL, CO, JK, DI, FY blood-group-encoding genes (20 fragments). RHCE and RHBD− genomic DNA samples have been amplified with both MPX PCRs and the products run out on 4 to 20 percent polyacrylamide gels.
Several reports from North America and Europe have indicated the potential of DNA arrays for blood group genotyping in the past 3 years. Our study, when completed, will represent a large cohort of blood donors and patients tested for the largest number of alleles in one study. Perhaps blood group genotyping is not yet ready to replace blood group serology entirely. However, vast technical developments in molecular genotyping, as well as our developing knowledge of the high degree of polymorphism within the human genome sequence, makes it certain that molecular genotyping will be a fundamental technology to health-care professionals. This may well signal that the end of blood group serology as we know it is nigh.

REFERENCES

42. Olsson ML, Chester MA. Evidence for a new type of O allele at the ABO locus, due to a combination of the A2 nucleotide deletion and the Ael nucleotide insertion. Vox Sang 1996;71:113-17.
46. Wagner FF, Poole J, Flegel WA. Scnanna antigens including Rd are expressed by ERMAP. Blood 2003;101:752-7.
55. Ait Soussan ARD, Bonsel GJ, et al. Prenatal RHD testing of fetus and mother: decision to administer anti-D


