Review Article

An overview of blood group genotyping

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Abstract: Predicting blood group phenotypes from DNA sequence, an alternative method to conventional blood group serology, began shortly after the molecular genetic bases of many blood group polymorphisms were ascertained in the 1990s. A variety of platforms have now been developed and commercialised, and are discussed in this review. Blood group genotyping is usually applied when no suitable red cell sample is available, when genomic testing will provide more or better information than serological testing, or when genomic testing is more efficient or cost effective than serological testing. One important application is the determination of fetal D (RH1) phenotype from fetal DNA present in the mother's blood, to assess the risk of haemolytic disease of the fetus and newborn or the requirement for antenatal anti-D immunoglobulin treatment. Other applications are discussed, including the use of high-throughput genotyping technologies to make it possible to screen large numbers of blood donors for multiple clinically significant blood groups and their variants, to improve matching between donors and patients to provide a higher degree of precision medicine.

Keywords: Blood groups; genotyping; genomics; haemolytic disease of the fetus and newborn; fetal blood grouping

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Introduction

At the beginning of the 20th century, Landsteiner in Vienna discovered the ABO blood groups by noting diverse patterns of agglutination when red blood cells were mixed with plasma from different individuals. Now, over 120 years on, agglutination of red cells by antibodies in plasma or derived from cultured B cells remains the most common and definitive method of distinguishing blood groups, although agglutination enhancement methods, such as enzyme treatment of the red cells or bridging by secondary antibodies, are often required.

In 1986, *GYPA*, the gene encoding Glycophorin A, the protein expressing the MN blood group antigens and many other antigens of MNS system, was the first blood group gene to be cloned and sequenced (1). This was followed by *ABO*, the gene encoding the glycosyltransferases responsible for biosynthesis of the ABO antigens (2) and then *RHCE* and *RHD*, the genes encoding the antigens of the Rh blood group system (3-5). By 2021, 43 blood groups systems, containing a total of 345 blood group specificities, had been recognised by the International Society of Blood Transfusion (6). Most of these systems represent a single gene each, although four systems (MNS, Rh, Xg, Ch/Rg) represent two or three closely-linked homologous genes, making a total of 48 known blood group genes, all of which have been identified and the molecular genetic bases of all major blood group polymorphisms elucidated (7).

Some useful reviews on blood group genotyping are references (7-15).

What is blood group genotyping?

The term "blood group genotyping" is not generally used to refer to the determination of blood group genotypes, but rather to the prediction of blood group phenotypes from appropriate DNA sequences. Other terms used are molecular blood grouping and blood group genomic testing. Most blood group polymorphisms result from single nucleotide polymorphisms (SNPs) (6,7). Determination of

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the nucleotides in homozygous or heterozygous state, at the position of the SNP, will often predict the phenotype with a high degree of accuracy (usually 99%).

Why perform blood grouping by genotyping when there are serological methods available? There are three main reasons: when we need to know a blood group phenotype, but do not have a suitable red cell sample; when genomic testing will provide more or better information than serological testing; and when genomic testing is more efficient or more cost effective than serological testing.

One question often asked is how accurate is genotyping? The more appropriate question, however, is how accurately does genotyping predict a serological phenotype? Of course, this depends on the genotyping platform used and the level of accuracy required. In some cases, inaccurate results compared with phenotyping might occur when gene sequence changes separate from the SNP being tested affect antigen expression. For example, in the Kidd system, the antithetical antigens Jk^a (JK1) and Jk^b (JK2) result from c.838G>A in *SLC14A1*. Determination of the genotype at that position will predict Jk^a/Jk^b phenotype with a high level of accuracy, but undetected inactivating mutations in *SLC14A1* would give rise to false predictions, since the protein predicted to express Jk^a or Jk^b would not be present in the red cell. These mutations are rare in most populations and design of blood group genotyping platforms must take into account the population to be tested. For example, a rare splice site mutation (IVS5–1) in *SLC14A1* that prevents Jk^b expression is relatively common in Polynesians, with frequencies between 0.3% and 1.4% (16-18).

On the other hand, genotyping may predict the presence of an antigen that is expressed too weakly to be detected by serological methods with the reagents available, yet may still be of potential clinical importance. In this case, genotyping may be considered more accurate than serological typing. For example, the very weak Fy^b (FY2) antigen referred to as Fy^x is often not detected by serological tests, but is revealed by molecular testing (19).

Although most genotyping tests involve detecting variation in the genes encoding the antigen (e.g., Rh, Kell, Duffy, and Kidd systems), others involve detecting variation in genes encoding glycosyltransferases responsible for the biosynthesis of carbohydrate antigens [e.g., ABO (20)] or of regulator sequences controlling gene expression [e.g., P1 (21)].

On rare occasions mutations in genes other than the blood group gene may affect antigen expression. For example, homozygous inactivating mutations in *RHAG* results in Rh_{null} phenotype, mutations in *XK* affects expression of Kell-system antigens, and various mutations in the erythroid transcription factor gene *KLF1* affects expression of Lutheran and other blood group antigens (7). These mutations are likely to give rise to false results with all but the most sophisticated of blood group genotyping platforms.

The term "blood group genotyping" also covers genomic testing for human platelet antigens (HPA) (22) (https://www.versiti.org/hpa) and human neutrophil antigens (HNA) (23).

Applications of blood group genotyping

Blood group genotyping has a large variety of applications in transfusion medicine, obstetrics, and transplantation medicine. Some of those applications are summarised below and listed in *Table 1*.

Genotyping is used to determine blood groups extended beyond ABO and D on recently transfused patients, where it is not possible to do the testing serologically because of the presence of transfused red cells. These are usually transfusion-dependent haemoglobinopathy patients. Although these patients should receive full serological testing before commencement of the transfusion programme, this does not always occur. Knowledge of the patients' extended blood groups means that matched blood can be provided in an attempt to prevent them from making multiple antibodies (24).

Genomic testing can be used for determining blood group phenotypes on red cells that have been coated with immunoglobulin *in vivo* and give a positive direct antiglobulin test (DAT), making serological testing difficult. This is particularly useful in helping to identify underlying alloantibodies in patients with autoimmune haemolytic anaemia.

Serological testing may be compromised in patients undergoing treatment with therapeutic monoclonal antibodies, especially anti-CD38 (daratumumab) and anti-CD47, which bind red cells (25-27). Consequently, genotyping is useful for blood grouping these patients.

Genomic methods can be used for defining the numerous variants of D, so-called weak D and partial D, to assist in making decisions about how to transfuse these patients, ensuring that those capable of making anti-D receive D– blood, but without wasting valuable D– donor blood on those patients unlikely to make anti-D (28). Genomic D-variant testing can reduce the unnecessary treatment with anti-D immunoglobulin of pregnant women with a D-variant red cell phenotype, but who are very unlikely to

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Table 1 Some applications of blood group genotyping

Blood group testing on patients who have recently been transfused
Blood group testing of patients whose red cells are coated with immunoglobulin *in vivo* (DAT+)
Blood group testing of patients being treated with therapeutic monoclonal antibodies.
Determination of D (RH1) variants in patients
Determination of RhCE variants in patients
Screening apparent D– donors for weak expression of D
Testing patients for multiple clinically significant blood groups and their variants
Blood grouping when serological reagents are rare or unreliable
Preimplantation genetic diagnosis for avoidance of HDFN
Assistance with identification of blood group antibodies in the reference laboratory
Screening donors for multiple clinically significant blood groups and their variants
Determination of *RHD* zygosity
ABO typing from buccal swabs in transplantation registries
A_1/A_2 typing in solid organ donors
Determination of fetal blood group to assess risk of HDFN
Determination of fetal blood group to assess requirement for anti-D immunoglobulin

make anti-D following a D+ pregnancy (28). Genotyping is also valuable for defining RhCE variants. Such variants are relatively common in people of African origin and their identification can help in finding suitable donors for sickle cell disease patients to reduce antibody production (29).

Another application is screening apparent D– donors for the presence *RHD*, in order to confirm that they do not have a weak form of D, such as the extremely weak DEL antigen, which goes undetected in standard serological tests, yet might still be able to immunise a D– patient or boost a pre-existing weak anti-D (28,30). Routine genomic screening of all serologically D– donors is provided by some blood services (31-33).

Preimplantation genetic diagnosis can be used for avoidance of HDFN when a blood group antibody, which has already caused severe or fatal HDFN, is present in a woman whose partner is heterozygous for the allele encoding the culprit antigen. Following *in vitro* fertilisation, single blastomeres from cleavage-stage embryos can be genotyped and only those that are predicted to be antigen negative would be implanted (34,35). This technology has only rarely been applied.

The most common genetic background to the D– phenotype is homozygosity for a deletion of *RHD* (7). Genomic methods can be used for determining whether a D+ person has one or two copies of *RHD* (i.e., hemizygous or homozygous), which cannot be done with any accuracy by serological methods. Zygosity testing may be achieved either by detecting a hybrid of the two *Rhesus boxes* that flank *RHD* and is only present when *RHD* is deleted (36) or by quantitative methods that distinguish one or two copies of *RHD* (37-39). Zygosity testing is potentially useful for testing fathers of fetuses at risk from HDFN because the mother has anti-D: if the father is homozygous for *RHD*, then the fetus must be D+ and there is no need for fetal testing. When non-invasive fetal testing is available, this test is seldom necessary.

Genotyping can replace serological tests that are unreliable or when suitable antisera are unavailable: for example, Do^a (DO1) and Do^b (DO2) antibodies are rare and unreliable; Fy^b (FY2) testing, as the presence of a weak Fy^b antigen (Fy^x phenotype) may not be detected by some antibodies; and Js^a (KEL6) testing of donors for patients with anti-Js^a, as anti-Js^a reagents are generally not available.

Genomic methods are extremely useful in the serological reference laboratory in helping to solve difficult problems. In the identification of unusual antibodies, exome sequencing (by next-generation sequencing) for all known

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blood group genes will reveal unusual genotypes of the maker of an antibody that will give valuable clues to the antibody specificity.

In many blood services the majority of blood donors are only tested for ABO and D, with a small proportion screened for multiple clinically-significant blood groups. Automated serological testing for this extended blood grouping is commonly being replaced by DNA testing. The advantages of genomic testing over serology are that it is more suited to high-throughput automated testing, more accurate, and identifies some phenotypes that cannot be tested for by serology. Extended blood group testing is essential for finding matched blood for patients requiring chronic transfusion support to prevent them from making multiple antibodies, or to find compatible blood for patients who already have blood group antibodies (40-44). Extended blood grouping of donors generally requires testing for M/N S/s U U^{var}, Rh C/c E/e hr^s hr^b and other Rh variants, K/k, Fy^a/Fy^b and GATA mutation, and Jk^a/Jk^b. In addition, tests for antigens of the Kell (Kp^a/Kp^b, Js^a/Js^b) and Dombrock (Do^a/Do^b, Hy, Jo^a) systems, plus Lu^a/Lu^b, Di^a/Di^b, Yt^a/Yt^b, Sc1/Sc2, and Co^a/Co^b, may be included.

The diversity within the Rh system in people of African origin may contribute to the high number of Rh antibodies in patients with sickle cell disease, which often makes the provision of compatible blood extremely difficult. For example, 6% of D+ and 21% C+ African Americans have partial D and partial C, respectively, and may make anti-D or anti-C following transfusion (42). Screening donors for Rh variant phenotypes is important for the provision of this rare blood and can only be achieved by genomic testing (41,43).

Antibodies to HPA may be involved in platelet refractoriness leading to failure of platelet transfusions, thrombocytopenia, and bleeding. Genotyping is the usual method for determining HPA phenotype and many platforms that test for multiple blood groups also include HPA testing (HPA1 to HPA9, plus HPA11 and HPA15) (9,44-50).

Antibodies to the five systems of HNA have been implicated in transfusion-related acute lung injury (TRALI), alloimmune and autoimmune neutropenia, and refractoriness to granulocyte transfusions (23). Immunological testing for HNAs has now mostly been replaced by molecular testing (apart from HNA-2, owing to a gene expression defect) (15).

In transplantation medicine, ABO genotyping may be used by transplant registries, which often collect buccal swabs, but not red cells. In addition, ABO genotyping may be used for confirming A_2 phenotype of solid organ donors, since A_2, but not A_1, organs are often considered suitable for group B patients (9).

Another important application of blood group genomics, predicting the blood group phenotype of a fetus, will be discussed below.

Technology involved in blood group genotyping

In addition to the cloning and sequencing of blood group genes and the identification of the nucleotide changes responsible for blood group polymorphisms, the technology that made blood group genotyping feasible in non-specialist molecular genetics laboratories was the polymerase chain reaction (PCR). This made it possible to analyse the DNA sequence of a small region of a blood group gene, from a small quantity of total genomic DNA.

When it was discovered that the D– phenotype in Caucasians nearly always results from a total deletion of *RHD*, it was readily apparent that D phenotype could be predicted simply by determining whether *RHD* was present (51). This is done by PCR amplification of one or more regions of *RHD*, with primers designed so that they only amplify *RHD* and not the homologous *RHCE*. Inactive *RHD* and *RHD-RHCE-RHD* hybrid genes, which, despite containing *RHD* sequences, produce no RhD antigen, complicate the methodology, but can be accommodated by the careful selection of PCR primers (52,53).

Most other blood group polymorphisms are encoded by SNPs (6,7). A variety of methods has been employed for distinguishing allelic single nucleotide alternatives in PCR products. Traditional methods involve PCR amplification of the region containing the SNP, followed by digestion of the PCR product with restriction enzymes, or by carrying out PCR in which one of the primers is designed to initiate amplification from only one of the alleles (54). Several commercial kits became available for this sort of testing (47). These methods are not generally high-throughput and often require gel electrophoresis, which increases contamination risks. Another traditional method is to carry out a PCR of the region containing the polymorphism, then sequencing the products by automated Sanger sequencing. Direct sequencing is low throughput and expensive, though it does give additional information about nucleotides around the SNP that might affect antigen expression.

Allelic discrimination by quantitative PCR with Taqman technology is generally read on a real-time PCR machine. For each polymorphism a pair of fluorescent probes is

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employed, each specific for each of a pair of alleles and each carrying a different reporter dye. These probes anneal to DNA of the appropriate sequence and are only released during amplification. Only then can they fluoresce under a laser. Relative quantities of PCR product for each allele can then be compared by computer (55). The multiplex reactions can be analysed on plates that contain 3072 holes (56) and this technology is readily adaptable for automated testing. In the multiplex ligation-dependent probe amplification assay, ligation of contiguous probes is allele-specific. Only ligated probes are amplified and fluorescently labelled, and, therefore, detected in a genetic analyser (57).

DNA arrays are chips or beads that have many short DNA sequences attached (45,58,59). Multiplex PCR amplifications carried out on the DNA of the subject provide fluorescent amplification products of all regions containing the polymorphisms to be tested. The amplification products are then incubated with a microarray or with microbeads of a variety of colours coated with oligonucleotides representing complementary sequences of all the polymorphisms to be tested. After scanning of the array for fluorescence by a laser scanner, or passing the beads through a laser, the results are analysed by a computer. Several commercial applications for blood group genotyping involving variations of this technology have been developed (47). Since the application of next generation sequencing (see below), array analysis has become more sophisticated. Application of DNA microarrays that were used in a transfusion medicine genome-wide association study has enabled the development of a high-throughput universal blood donor genotyping platform capable of simultaneously typing all clinically relevant blood group antigens and their variants, plus platelet, granulocyte, and leukocyte (HLA) antigens (44,50).

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has also been adapted for blood group genotyping (46). Biomolecules are ionised by a nanosecond laser pulse and the ions are accelerated in an electric field along a flight tube. Molecules are separated according to their mass:charge ratio and reach a detector at different times. DNA fragments differing by only a single nucleotide can be differentiated by their time of flight, distinguishing alleles.

Next-generation sequencing, also called massively parallel sequencing or, for long reads, third generation sequencing, is an extremely powerful technology designed originally for sequencing the whole genome. Next generation sequencing provides the capacity for rapid sequencing of the whole genome or whole exome, but also to sequence limited regions of the genome of many different individuals in one run, a potential for testing all required blood groups of numerous donors. Millions of sequencing reads can be obtained in a single run and are interpreted by high-volume informatics (60). Targeted enrichment, in which selected regions are enriched in a DNA library, permits analysis of all red cell blood group polymorphisms and the identification of variant genotypes, including those responsible for null phenotypes, plus all platelet antigens, by comparing sequences with those of reference sequences (9,13,41,49,61-63). Next generation sequencing technology, however, has been considered excessively expensive for extended blood grouping on large numbers of blood donors compared with microarray technology (44).

Fetal blood group genotyping

Despite widespread anti-D immunoglobulin prophylaxis programmes, some D– women still make anti-D. It is valuable to know the D type of the fetus of a woman with anti-D for appropriate management of the pregnancy: if the fetus is D+ it is at risk of HDFN and the pregnancy should be managed accordingly; if it is D– it is not at risk and there is no need for any intervention (10). In many countries, fetal *RHD* genotyping is now considered the standard of care for pregnancies at risk from HDFN. In the USA this application has been hampered by patent and licensing issues (9).

Initially, fetal DNA for *RHD* testing, first reported in 1993 (64), was obtained by amniocentesis or chorionic villus sampling. Both interventions are invasive and can lead to miscarriage or fetal haemorrhage with consequent boosting of anti-D. In 1997, discovery that cell-free fetal DNA is present in the plasma of pregnant women provided a non-invasive source of fetal DNA (65). At 10 to 20 weeks of gestation, a mean of around 10 to 14% of cell-free DNA in maternal plasma is of fetal origin (the fetal fraction), but the range is large, with some pregnant women having a fetal fraction of <1.5%. After 21 weeks the proportion of fetal DNA increases by about 1% per week (66,67). Fetal DNA cannot be isolated from the maternal DNA, but since the mother must be D–, as the reason for testing is that she has made anti-D, she will usually have no *RHD*. Therefore, if *RHD* is detected it must be of fetal origin and the fetus must be D+, whereas if no *RHD* is detected the fetus must be D–. Most methods for fetal *RHD* detection involve testing for two or more regions of *RHD* to avoid false positive results arising from variant *RHD* genes that produce no D antigen.

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The usual technology employed is real-time quantitative PCR (QPCR) with Taqman chemistry, which measures the quantity of amplified product at every cycle (68). The quantitative aspect of this technology ensures that only fetal DNA is being amplified, not the much larger quantity of maternal DNA. It is possible to include various fetal markers to control for the presence of sufficient fetal DNA and successful amplification when the fetus is D–, but none is entirely satisfactory or cost-effective so, considering the very high level of specificity demonstrated across several studies, internal positive controls are not generally used routinely (10,11,69).

Fetal testing on fetal DNA from maternal plasma in alloimmunised pregnant women is also often provided for C (RH2), E (RH3), and c (RH4) by testing *RHCE*, and for K (KEL1) by testing for a SNP on *KEL*. Antibodies to these antigens can cause severe HDFN, particularly anti-c and anti-K. Tests are generally carried out by QPCR with an allele-specific primer or probe (70). Most studies from various laboratories report 100% accuracy for C, E, and c, but a few errors for K, which has proved more challenging (11,70).

To prevent D immunisation during pregnancy, it is common practice for all D– pregnant women to be offered anti-D immunoglobulin prophylaxis at about 28 weeks of pregnancy. This is in addition to that given after delivery of a D+ baby. Without fetal testing, this antenatal treatment must be offered to all D– pregnant women, yet in a Caucasian population up to 40% of these D– pregnant women will have a D– fetus and receive the treatment unnecessarily. [In African populations the frequency of D– is substantially lower and in East Asians D– is rare (7)]. In several European countries, including Denmark, the Netherlands, Sweden, England, France, and Norway, routine non-invasive genomic D testing on fetal DNA obtained from the maternal plasma is being offered to all D– pregnant women (11,71-75). This testing has a very high level of sensitivity and specificity from 11 weeks gestation and eliminates unnecessary treatment of pregnant women with anti-D immunoglobulin and the associated inconvenience, discomfort, and perceived risks of infection by unrecognised viruses or prions. Fetal *RHD* screening is cost effective, with the expense of the test offset in several ways: by savings in the cost of antenatal anti-D immunoglobulin given at 28 weeks gestation and following potential sensitising events; by a decrease in fetal haemorrhage testing; by maximising hospital bed capacity by enabling midwives to give anti-D immunoglobulin immediately after the delivery of a D+ baby without having to wait for laboratory results; and, in some countries, by discontinuation of cord blood typing (11,74). In addition to these benefits, there is a worldwide shortage of anti-D immunoglobulin, which is produced in volunteers who have been immunised with blood products, so there are ethical issues around wastage of this valuable gift (76,77).

Although QPCR remains the method of choice for routine fetal blood group testing, some proof of principle studies with targeted next generation sequencing have demonstrated that this technology may be applied in the future (78-80). Advantages of next generation sequencing for fetal testing are that it can determine fetal fraction and that analysis of multiple fetal sequences eliminates concerns about absence of positive controls (80).

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) results from maternal platelet antibodies, usually anti-HPA1a, crossing the placenta and destroying antigen-positive fetal platelets. At its most severe, FNAIT causes intra-cranial haemorrhage, frequently leading to fetal or neonatal brain damage and death (81). Tests involving QPCR, high-resolution melting analysis, or next generation sequencing have been developed for determining fetal HPA1a type from cell-free fetal DNA obtained from the plasma of pregnant women with anti-HPA1a (14,82,83).

External quality assurance (EQA)

When blood group genotyping is being used for clinical purposes, it is important that it is properly regulated. This regulation should include participation in an EQA programme. Initially the International Society for Blood Transfusion (ISBT) provided a series of four workshops (2004–2010) that functioned as EQA exercises and included DNA samples for multiple blood group typing plus two samples from pregnant women for fetal D typing (84-87). An EQA scheme was also provided by INSTAND in Germany (88). UK NEQAS launched a pilot genotyping EQA scheme comprising four exercises per year in 2016/17 with the aim of becoming a full UK NEQAS EQA Scheme in 2020/21 (89). These exercises have revealed a high level of accuracy achieved with a variety of different platforms, but an unacceptable diversity of blood group genotype and phenotype nomenclature.

Four workshops comprising about 28 laboratories have been organised in Denmark since 2016. Each participant tested two blood samples from pregnant women, one with a D+ fetus and one with a D– fetus: no false-negative or false-positive results were reported (90). All participating

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laboratories used QPCR. These workshops will continue as an annual event and will be organised by DEKS EQA (https://deks.dk).

What about the future of blood grouping?

A question commonly asked is will genotyping replace serological testing for blood grouping? For routine donor and pre-transfusion ABO testing I think that the answer is no, at least in the foreseeable future. The reason for this is that ABO testing is relatively simple to perform serologically and is exceptionally reliable, whereas ABO is highly complex genetically. And for ABO testing, there is no margin for error. For pre-transfusion D testing the same reasoning probably applies and it may be a long time before serological D testing will be replaced by genotyping. It is likely that all other blood group antigen typing will soon be performed by genotyping, at least in some countries. Antibody screening and identification will still require serological methods, but much of this may be done with synthetic, recombinant antigens (91) or genetically-modified cultured red cells, for example, cells created by CRIPR-mediated gene editing of an immortalised human erythroblast cell line (92,93).

If all donors and patients could be tested for all clinical important blood groups, rapidly and at relatively low cost, then electronic matching of donors to patients would be feasible. This would result in a decrease in levels of alloimmunisation, reducing haemolytic transfusion reactions, especially delayed transfusion reactions where antibodies are not detectable serologically owing to evanescence of the antibody. It would also save time and expense involved in complex serological investigations. This will be possible within the near future from the point of view of blood group testing, but the logistics of obtaining matched blood for the right patient will be more complex. If there is a will to deliver this level of precision medicine, then a way can be found.

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