Chapter

Accuracy of Blood Group Typing in the Management and Prevention of Alloimmunization

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Abstract

Blood transfusion is an effective therapeutic approach for several hematological conditions including sickle cell disease (SCD), thalassaemia, myelodysplastic syndrome (MDS), and autoimmune hemolytic anemia. It is also often indicated for transplantation and for patients receiving medical treatments for cancer. However, transfusion treatment can lead to the red blood cell (RBC) alloimmunization when an incompatible antigen is inadvertently present in the transfused blood. Alloantibodies can cause RBC destruction and many other complications defeating the purpose of the treatment. The risk of development of multiple alloantibodies increases with the frequency of transfusions in transfusion-dependent patients and can be mitigated by transfusing blood type negative for multiple antigens to prevent hemolysis. This chapter discusses the transfusion's risk of RBC alloimmunization as an adverse event; consequences of alloimmunization in patients' care; approaches to prevent and/or mitigate alloimmunization and enhance transfusion efficacy; application of RBC genotyping to supplement serology for preventing alloimmunization. The currently available techniques for RBC genotyping and the importance of reference reagents for determining the genotyping accuracy will also be discussed.

Keywords: blood transfusion, RBC alloimmunization, blood groups, blood group genotyping, reference materials

1. Introduction

Blood transfusion saves lives in trauma situations and serves as a unique therapeutic treatment of various hematologic disorders where patients require lifelong regular transfusions to survive. However, in contrast to therapeutic drugs, blood cannot be manufactured and tested for quality control of purity, potency, and efficacy. Instead, each donation is a unique batch, most often single use, for which all characterization and quality assessment need to be performed individually even if several units are used in a single treatment. Since blood is a biological product, safety assessment must be done at multiple levels, including donor screening, blood collection, testing for infectious agents, blood cell typing and labeling. Checkpoints and quality control are also required at the time of transfusion to avoid human error and to guarantee that the right blood goes to the right person. Each step has its own characteristics and challenges. Screening involves the review of donor history that allows assessment for safety; requirements that need to be followed for blood collection to assure proper timing, storage, and processing so that the quality of the

product is not compromised; testing for infectious agents ensures a second layer of screening for pathogens that could be transmitted to the patient with an infected blood unit; testing for blood cell typing helps to avoid improper usage of the product since non-compatible blood can produce serious consequences and even fatalities; lastly, the transfusion process itself requires strict checkpoints to prevent the use of an incompatible unit in a given patient.

The scope of this chapter focuses on blood group typing and approaches to achieve better management and prevention of alloimmunization. We will provide an overview on the following topics: blood transfusion and risk of RBC alloimmunization; approaches for prevention of alloimmunization and improvement of transfusion therapy; serology and molecular typing methods and their use for RBC matching; current technologies for RBC genotyping; and standards for red cell antigens typing including scope, importance, and description of reference reagents available for use.

2. Blood transfusion and risk of RBC alloimmunization

RBC transfusion is used for therapeutic treatment of various hematologic disorders including SCD, thalassaemia, MDS, autoimmune hemolytic anemia, and others. Management of patients may require lifelong regular transfusions for treatment of symptomatic anemia and prevention of disease complications. Although transfusion safety and donor-patient matching have improved over the years, RBC alloimmunization, transmission of infections, and iron overload are still a concern [1].

RBC alloimmunization is a serious adverse event of transfusions and can cause further clinical problems in the patients including worsening of anemia, development of autoantibodies, acute or delayed hemolytic transfusion reactions (DHTR), bystander hemolysis, organ failure, and cause serious complications during pregnancies. Frequent transfusions can lead to the production of multiple alloantibodies, which is often associated with autoantibodies requiring extensive serological workups and additional transfusions for proper treatment, increasing time and resources to find compatible RBC units [2].

Antibodies against ABO blood group antigens are naturally produced, IgM class, and are capable of rapid induction of intravascular destruction of RBCs by complement-mediated mechanism. Clinical outcome of a patient transfused with incompatible ABO blood can vary from no adverse effects to permanent organ damage and death depending on the volume of ABO-incompatible RBC transfused. As for non-ABO blood groups, clinically significant antibodies against non-ABO antigens are from IgG class and rarely activate complement. Instead, they cause DHTR or hemolytic disease of fetus and newborn (HDFN) by extravascular destruction of RBCs marked with IgG [3]. DHTR has been ranked as second or third most commonly reported cause of fatal transfusion reactions secondary to non-ABO antibodies in many countries including the United States [4–8]. However, experts believe that DHTR has been under-recognized or under-reported to biovigilance agencies and FDA, possibly because the reactions can be obscured by underlying disease (liver disease, massive trauma, and SCD), lack of knowledge among clinicians regarding the reactions, or lack of knowledge regarding the mechanism of reporting [7].

Reported RBC alloimmunization rates have considerable variations depending on the population and disease studied [9]. The rates are estimated between 1 and 3% in patients that receive episodic transfusions, while for patients who receive chronic blood transfusions like patients with SCD and MDS, rates vary between 8 and 76% [9–12]. Although the most commonly observed alloantibodies of clinical

relevance are against antigens belonging to RH (D, C, c, E, e), KEL (K, k, Js^a, Kp^a), JK (Jk^a, Jk^b), FY (Fy^a, Fy^b), and MNS (M, S, s) blood group systems [9], alloantibodies against Rh variants [13–15] and other rare blood group phenotypes have also been implicated in shortened survival of transfused RBCs by causing DHTR [13, 16] or HDFN [17]. In addition, some antibodies only have occasional reports of being clinically significant, that is, anti-Yt^a, -Ge, and -N or have no clinical significance unless reactive at 37°C, that is, anti-Le^a, -Le^b, -M, -N, -P1, -Lu^b, -A1, and -Bg [18].

The development of RBC antibodies is influenced by many factors including recipient's gender, age, and underlying disease. The diversity of the blood group antigen expression among the donor and patient populations contribute substantially to the high alloimmunization rates [15]. Other factors that might be associated with alloimmunization risk have been actively explored in seeking novel strategies for prevention of alloimmunization [19]. Studies in both animal models and SCD patients have reported that inflammation is associated with higher likelihood of alloimmunization and it is suggested that the extent of the alloimmune response is higher when RBCs are transfused in the presence of an inflammatory signal [19–23]. Several studies have suggested that genetic variation in immune-related genes such as *TNFA*, *IL1B*, *CTL4*, *CD81*, *TRIM21*, *TLR1/TANK*, *MALT1* [24–28], and human leukocyte antigens (HLA) [24, 29] might be associated with susceptibility to or protection from alloimmunization. Importance of unit's age and blood product modifications like leukoreduction or irradiation has also been investigated but their impact remains unclear.

3. Prevention of alloimmunization and improvement of transfusion therapy

Prevention of alloimmunization is desirable for any blood transfusion; however, for patients not previously transfused or only having episodic blood transfusions, matching for all clinically significant antigens is not of great concern, but can result in alloimmunization against non-matched antigens. For patients previously transfused, particularly transfusion-dependent patients, the alloimmunization risk is higher and management of alloimmunized patients is of greater concern. Their alloimmunization status, including antigens of low clinical significance, is a critical part of their clinical history that may enable health care providers to take measures to prevent further alloimmunization.

As of May 2019, the International Society of Blood Transfusion (ISBT) recognizes 36 blood group systems. Of the 360 identified antigens, 322 have been assigned to a specific blood group system. These antigens have variable immunogenicity and not all blood group antigens are involved with the production of clinically significant antibodies after blood transfusion or pregnancy. Ideally, every blood transfusion should be compatible for the most clinically significant antigens in the RH (D, C, E, c, e), KEL (K), FY (Fy^a, Fy^b), JK (Jk^a, Jk^b), and the MNS (S, s) systems to prevent alloimmunization; however, the standard pre-transfusion cross-matching is only performed for ABO blood group and the Rh(D) antigen; ABO matching is performed to avoid acute hemolytic transfusion reactions caused by natural IgM antibodies against ABO antigens, and Rh(D) matching is performed because of the high immunogenicity of the Rh(D), which is implicated in DHTR and HDFN.

Currently, recommendations for partial and extended donor unit/patient matching are limited to specific groups including [1] patients on long-term transfusion protocol (i.e. SCD, MSD, thalassaemia, and aplastic anemia), [2] patients who have developed alloantibodies, and [3] patients with warm autoimmune hemolytic anemia.

Verification of compatibility for Rh (D, E, C, c, e) and K, which are the most frequent antigens involved in alloimmunization, is considered partial matching. Extended matching should include at least RH (D, C, E, c, e), KEL (K), FY (Fy^a, Fy^b), JK (Jk^a, Jk^b), MNS (S, s) and, if available, additional antigens.

3.1 Serology typing

Knowledge of the role of blood groups with their antigens and variants in alloimmunization was pivotal for the development of transfusion practices and medical interventions that require blood transfusion such as trauma, hematological diseases (e.g. SCD, MDS, thalassaemia, and aplastic anemia) and later for transplantation and cancer treatment.

Serology has been considered the gold standard technique for blood group typing for a long time. Serological methods detect the antigen expressed on the red cell using specific antibodies and can be carried out manually or by automated platforms. Typing of blood group antigens using this method is easy, fast, reliable, and accurate for most of the antigens. However, serology has limitations, some of which cannot be overcome when it is used as a standalone testing platform (**Table 1**). Scarcity of serological reagents for some blood group systems for which there is no monoclonal antibody available is a major limitation. In addition, human serum samples from different donors vary in reactivity, which is an issue when a nearly exhausted batch of reagent needs to be replaced. This is especially problematic when an alloantibody for that antigen is suspected to be causing adverse events after transfusion. In those circumstances, molecular methods can be used as an alternative or as a complementary test for identification of genes associated with the blood group antigens expression and prediction of antigenic profile (see **Table 1**).

3.2 Molecular typing

The identification of genes that encode proteins carrying blood group antigens and the molecular polymorphisms that result in distinct antigenicity of these proteins is possible using molecular typing methods, which facilitate blood typing

Serology limitation	**Genotyping application**
No available antisera, weak or limited antisera (i.e. Do^a, Do^b, Js^a, Js^b, Kp^a, Kp^b, V and VS)	Blood group typing can be easily performed by single PCR and/or high throughput platform
Mixed field caused by the presence of donor's RBCs in patient's sample (i.e. patients with recent blood transfusions) Presence of interfering antibodies (i.e. autoantibodies, multiple antibodies, antibodies against high prevalence antigens)	Genotyping is performed with extracted DNA from nucleated cells (e.g. leukocytes, epithelial). The presence of donor's RBCs or interfering antibodies in patient's sample does not interfere with the results
Presence of variant antigens (i.e. hybrid RH types, FY silencing mutations, MNS hybrids)	Detection of genes and molecular mechanisms associated with variant antigen expression
Detection of blood type of fetus at risk of HDFN without invasive procedure	Detection of blood type and zygosity on DNA extracted from maternal plasma
Mass screening for antigen-negative and rare RBC phenotypes	Use of high throughput platforms for donor screening allows mass scale typing and creation of databases

Table 1.
Benefits of genotyping over serology.

resolution in complex cases and overcome limitations of serological techniques when dealing with alloimmunized and multitransfused patients. In addition, molecular techniques have allowed identification of genes encoding clinically relevant antigens for which serological reagents are not available (see **Table 1**). In those instances, genotyping is critical to resolve clinical challenges.

Blood group genotyping is performed to predict blood group antigens by identifying specific polymorphisms associated with the expression of an antigen. Most variations in the blood group antigens are linked to point mutations, but for some, other molecular mechanisms are responsible, such as deletion or insertion of a gene, an exon or a nucleotide sequence (i.e. ABO, RH, and DO blood group systems), sequence duplication, (i.e. RHD gene and GE blood group system), nonsense mutation (i.e. RHD gene), and hybrid genes (i.e. RH, MNS, ABO, and CH/RG blood group systems) [30].

In contrast to serology, molecular tests are performed on DNA obtained from nucleated cells and are not affected by the presence of donor's red cells in patient's sample, which is a common occurrence in samples of patients with recent/multiple blood transfusions. Thus, RBC genotyping can resolve blood group typing discrepancies in multitransfused patients presenting with mixed field reactions, alloantibodies, or autoantibodies (**Table 1**). Also, blood group genotyping can substantially help patients who were not previously phenotyped and need regular transfusions by facilitating management of these patients and preventing alloimmunization [31].

Studies comparing serology and genotyping in multitransfused population such as patients with thalassaemia and SCD have shown that genotyping is superior to serology for resolving discrepancies [31–35]. Use of genotyped matched units has been shown to decrease alloimmunization rates [36], increase hemoglobin levels and in vivo RBC survival, and diminish frequency of transfusions [37–39].

3.3 Sickle cell disease

SCD is the most common congenital red blood cell disorder affecting millions of people worldwide with high mortality and morbidity rates [40]. It is considered a major public health issue by the WHO. Characterized by an abnormal synthesis of hemoglobin, this genetic trait is most common among people of African ancestry. Abnormal hemoglobin carried in red cells causes these cells to sickle (thus the name SCD), which as early symptoms produces swelling of the hands and feet, anemia, fatigue, and jaundice. Long-term effects of the disease include serious damage in spleen, brain, eyes, lungs, liver, heart, kidneys, bones, and/or skin that can accumulate over a person's lifetime. Patients can survive beyond their 50s, and most fatalities are not associated with chronic organ failure but occur due to an acute episode of one of the SCD complications. SCD can be cured by bone marrow transplantation, but only a few patients get transplant.

Blood transfusion therapy is part of treatment for SCD patients and it is mainly indicated for prevention of stroke and vaso-occlusive crisis. However, transfusion benefits are limited upon development of alloantibodies, a common adverse event of transfusion. The high incidence of RBC alloimmunization in SCD is multifactorial, but lack of blood group compatibility between donor and recipient is a key factor. This is more evident in countries where donors' and patients' ethnicities significantly diverge, that is, in North America, where blood donors are mostly Caucasians while SCD patients are predominantly of African descent; thus, SCD patients are frequently exposed to foreign antigens and, consequently, have higher risk of developing multiple alloantibodies.

Transfusion protocols for management of SCD and prevention of alloimmunization vary among the hospitals and transfusion services. In many centers that provide care to SCD patients, transfusions are phenotypically matched for RH

(D, C/c, E/e) and K [41], while others provide extended matching including RH (D, C, c, E, e), KEL (K, k), FY (Fy^a, Fy^b), JK (Jk^a, Jk^b), and MNS (S, s) in addition to the standard ABO and Rh(D). Less frequently, extended matching is performed by genotyping [36]. However, a wide range of institutions do not request phenotypically matched RBC units until the patient has produced an alloantibody [42].

It has been reported that antibodies against Rh antigens are the most frequently identified antibodies in multitransfused SCD patients despite transfusion from Rh phenotype matched donors [15]. The main reason for that is the high frequency of Rh variants in people of African descent. It has been reported that 90% of SCD patients and donors of African ancestry have at least one variant *RHD* or *RHCE* allele [15, 43]. The term "variant" is used when *RHD* and/or *RHCE* genes are carrying genetic alterations that may affect the RhD and RhCe protein expression. Variant alleles can encode weak and/or altered antigens and serological methods are limited in which variant Rh antigens can be identified and might not be reliable [44, 45].

The prophylactic RBC matching performed by serology typing, before exposure to RBC transfusions, can decrease transfusion complications in SCD patients substantially, but does not eliminate the occurrence of alloimmunization against Rh variants and other non-matched antigens that can cause DHTR [41]. Currently available molecular typing methods can predict several blood group antigens allowing a more precise RBC matching and can support transfusion decision-making. *RHD* and *RHCE* genotype matching particularly benefits SCD patients carrying Rh variants. For instance, SCD patients presenting D+ or e+ phenotype can make alloantibodies to these antigens despite receiving Rh phenotyped matching RBCs. The molecular analysis in such individuals may identify polymorphisms in *RH* genes responsible for the phenotypic alteration, confirming the alloimmune status of the antibody. In such cases, provision of *RH* genotype matched units or units negative for the specific antigen would be recommended, because the antibodies produced may be clinically relevant [13, 15].

An additional benefit of blood group genotyping on transfusion management of SCD patients is the capability of identifying silence mutations like −67T>C in the ACKR1 gene (Duffy gene). Patients carrying the mutation can receive Fy(b+) units, because the mutation only abolishes expression of Fy(b) on red cells but not in other tissues. The detection of this mutation avoids unnecessary use of Fy(b−) and increases the chances to find compatible units available even for highly restrictive matching.

Extended genotyping including Dombrock: Do^a, Do^b, Jo^a, Hy; Kell: Kp^a, Kp^b, Js^a, Js^b; Rh: V, VS; Colton: Co^a, Co^b; Cartwright: Yt^a, Yt^b; Lutheran: Lu^a, Lu^b; Diego: Di^a, Di^b; and Scianna: Sc1, Sc2 may help prevent development of clinically significant antibodies that can be potentially life-threatening.

3.4 Thalassaemia

Thalassaemia is an inherited blood disorder associated with a mutation in one of the genes involved in hemoglobin production resulting in abnormal form or an inadequate amount of hemoglobin. RBCs carrying abnormal hemoglobin do not function properly and are destroyed in large numbers leading to anemia. People with thalassaemia may have mild or severe anemia depending on the type of thalassaemia. Severe anemia requires regular blood transfusions to maintain the hemoglobin and RBCs levels, and to suppress the ineffective erythropoiesis but can lead to alloimmunization.

The prevalence of alloimmunization in patients with thalassaemia varies among geographical locations and may be related to the heterogeneity of population, transfusion exposure frequency, patients age, antigen matching policy, recipient related

factors, and other factors [46]. The most common alloantibodies reported in these patients are against RH (primarily anti-E and anti-C) and K, followed by antigens of the FY, JK, MNS, and other blood group systems. Development of autoantibodies is also commonly observed in these patients. A policy for RH and KEL matching been introduced worldwide and its effectiveness has been demonstrated by the decreasing rates of alloantibody and autoantibody formation [46–48].

The *Thalassaemia International Federation* guidelines for transfusion-dependent thalassaemia published in 2014 recommends that all patients should receive prophylactic ABO, RH (D, C, c, E, e), and KEL (K) matched transfusions identified either with serology or genotyping. In addition, it is indicated that antigen typing should be performed using molecular rather than serologic testing if the patient had received transfusions previously [49]. However, surveys have reported that there is still a lack of adherence to recommendations and a large variation in transfusion practices for thalassaemia and SCD patients among the health care systems [42]. Some of the challenges for transfusion centers include difficulty in obtaining a reliable antibody and transfusion history and the lack of standards regarding procedures for phenotyping and transfusion matching [42].

Molecular typing has been introduced in several centers to confirm extended blood group profiles obtained through serological methods, however, it is not routine yet [31, 32, 42]. This approach is particularly important for thalassaemia patients because these patients are transfusion-dependent and, in many circumstances, might have received transfusions at hospitals with different transfusion matching policies, therefore the phenotyping might not be reliable.

3.5 Rare type blood donor selection

Alloimmunized patients require transfusion of RBCs that are negative for a particular antigen. Historically, serology methods, which are labor-intensive and time-consuming, have been used to screen for antigen-negative units. However, the standard practice is likely to change with the high-throughput platforms for blood group genotyping being approved by regulatory bodies and becoming more widely used. High-throughput platforms allow identification of a higher number of antigens compared with serology, increasing the availability of blood characterized for clinically relevant antigens.

The implementation of RBC mass scale genotyping for donor screening has started in blood centers, especially in large collecting facilities [50–53]. The successful establishment of a blood group genotype database has already been accomplished aiming to fulfill antigen-negative requests, especially for SCD patients receiving regular transfusions, and to create an inventory of frozen red cell units with rare blood types [51]. The refereed database comprises 43,066 non-Caucasian blood donors genotyped for 32 single nucleotide polymorphisms, related to the expression of 42 blood group antigens. The report showed that within 4 years of starting RBC genotyping, the blood group antigen database generated on blood donors was fivefold larger than that obtained by serology methods over 30 years. In addition, most antigen-negative units requests to that center were met using exclusively the genotyping database.

Strategies for finding units to fulfill transfusion requests for SCD patients have included RBC genotyping of non-Caucasian blood donors and donors with altered Rh antigen expression [54, 55]. The genotyping selection of donors with a genetic background similar to that of SCD patients' increases the chances of finding compatible blood for these patients, including RH-genotype matching. However, the low percentage of blood donors with African ethnic background combined with the cost of genotyping are limiting factors for widespread use of extended RBC genotyping matching strategy.

4. Molecular testing: current technologies

Polymerase chain reaction (PCR) and further advancements in molecular methods such as DNA sequencing and recombinant DNA technology allowed elucidation of the molecular basis of most of the blood group antigens and subsequent use of molecular techniques employed for blood group genotyping initially as in-house assays or laboratory developed tests (LDT) and later as commercially available assays.

While molecular basis for many of the blood group systems is relatively straightforward, with two antithetical alleles differing by a single nucleotide polymorphism (SNP) and responsible for one of the two possible phenotypes, some blood group systems are very complex. RH blood group system has over 50 antigens described

Name/manufacturer	Principle	Number of polymorphisms/antigens identified
Immucor PreciseType HEA Molecular BeadChip Test[#,$] [56] BioArray Solutions	Multiplex PCR followed by hybridization of amplified DNA to probes attached to spectrally distinguishable beads and elongation	24 polymorphisms associated with 38 antigens plus phenotypic variants and Hemoglobin S
BioArray RHCE and RHD BeadChip[$] [57] BioArray Solutions	Multiplex PCR followed by hybridization of amplified DNA to probes attached to spectrally distinguishable beads and elongation	35+ *RHCE* variants; 80+ *RHD* variants
ID CORE XT[#,$] [58] Progenika Biopharma	Multiplex biotinylating PCR followed by hybridization to probes on color-coded microspheres	29 polymorphisms associated with 37 antigens and phenotypic variants
ID RHD XT[$] [59] Progenika Biopharma	Multiplex biotinylating PCR followed by hybridization to probes coupled to color-coded microspheres	6 *RHD* variants and HPA-1
Hemo ID DQS Panel[$] [60, 61] Agena Bioscience	Multiplex end-point PCR and single base primer extension	101 antigens associated with 16 systems, and 23 platelet and neutrophil antigens, in modules
HIFI Blood 96[$] [62] AXO Science	Automated multiplex PCR and microarray-based assay	15 polymorphisms associated with 24 antigens
RBC-FluoGene vERYfy eXtend[$] [63] inno-train diagnostik GMBH	Automated TaqMan-based assay	70 antigens and phenotypic variants associated with 12 systems
RBC-ReadyGene[$] [64] inno-train diagnostik GMBH	PCR-SSP-based assay	16 modules encompassing antigens and variant phenotypes within 13 systems
TaqMan genotyping on OpenArray [65, 66] BioTrove	Nanofluidic TaqMan assays performed in OpenArray plates	16, 32 or 64 assays (custom designed)
Pre-designed TaqMan assays Thermo Fisher Scientific	Real-time allelic discrimination PCR	Polymorphisms associated with 8 systems

[#] *FDA approved.*
[$] *CE mark certification.*

Table 2.
Commercial solutions available for blood group genotyping.

Name	Principle	Number of polymorphisms/ antigens identified
Multiplex PCR-single-strand conformation polymorphism (SSCP) analysis [67]	Denaturation of PCR product followed by fragment analysis in polyacrylamide gel	9 polymorphisms associated with 1 blood group
PCR-RFLP [68–74]	Amplification of target sequence followed by enzyme digestion (PCR-RFLP)	Varies
PCR-SSP [75–77]	Amplification of single alleles using allele-specific primers	Singleplex or multiplex
Multiplex-PCR [78, 79]	Amplification with multiple primers followed by gel electrophoresis	Polymorphisms associated with 6 exons of *RHD*
Quantitative multiplex PCR of short fluorescent fragments (QMPSF) [80]	Amplification of multiple targets using both specific and universal-FAM-labeled primers detected by electrophoresis	10 exons of both *RHD* and *RHCE*
Denaturing high-performance liquid chromatography (DHPLC) analysis [81]	Amplification followed by enrichment with a known sample and DHPLC analysis	Entire *RHD* gene
Multiplex ligation-dependent probe amplification (MLPA) [82]	MLPA probes hybridized to DNA target sequence followed by fragment amplification and detection	48 alleles encoding blood group antigens and 112 variant alleles of 18 blood groups
GenomeLab SNPStream [83]	Multiplex PCR followed by single-nucleotide extension and hybridization to complementary oligonucleotides	12 polymorphisms associated with 6 blood groups and platelet antigens
High-resolution melting analysis [84, 85]	PCR with gene-specific primers and probes followed by melting curve analysis	Various—customized
Single nucleotide primer extension minisequencing assay (SNaPshot) [86]	Multiplex PCR followed by SNaPshot reaction and fragment analysis	Various—customized
Microsphere-based array [87]	Multiplex PCR followed by hybridization to probes coupled to fluorescent microspheres	8 polymorphisms associated with 6 blood groups
Multiplex PCR and DNA microarray hybridization [88]	Chimeric primers (specific-universal tag) are used along with fluorescent-label universal primers followed by products hybridization to a DNA array	Gene fragments of 19 different RBC and platelets antigen systems
Sanger sequencing [89]	Classical DNA sequencing of single fragment using dye terminators	Various—customized
Next generation sequencing (NGS) [90]	Sequencing of entire genome or specific regions. Millions of small fragments of DNA are simultaneously sequenced	Various—customized

Table 3.
Other techniques developed for blood group genotyping.

to date, more than 500 alleles, and 2 highly homologous genes that, in addition to SNPs, insertions, and deletions, may have hybrid configurations. Other systems with more than 50 alleles currently identified include MNS, Diego, and Kell. Genotyping strategies are often based on a SNP altering a restriction site or punctuating otherwise perfectly matched sequence for primers or probe annealing site. These approaches may be suboptimal for designing genotyping assays of complex systems and fail to reveal rare variants. Next generation sequencing (NGS) is the only method that can reliably detect the exact genetic composition of the site making it possible to predict the phenotype.

Genotyping methodologies vary widely and include labor-intensive techniques that are best suited to test individual samples for limited number of polymorphisms (i.e. PCR-RFLP and PCR-SSP), high-throughput commercial kits that are relatively easy to use (i.e. real-time PCR and arrays), and methods that require specialized equipment to differentiate between alleles in multiple blood group systems at once (i.e. NGS). Most of assays described to date rely on enzyme-mediated DNA amplification at some point in their workflow and on sequence-specific primers or probes (**Tables 2** and **3**).

At the time of this writing, several platforms have been commercialized, but only two commercial assays have been approved by the US FDA. Immucor PreciseType by BioArray Solutions was approved in May 2014 and ID CORE XT manufactured by Progenika Biopharma was approved in October 2018. Due to less stringent requirements to obtain the European Conformity (CE mark), this certification has been granted to most of the commercial devices described here (see **Table 2**). Commercial assays not approved by the FDA can be labeled and utilized for research or investigational use only. Some commercial platforms (such as OpenArray or GenomeLab SNPStream) are used to run LDT assays to increase the throughput (**Table 3**).

5. Molecular testing: standards for RBC antigens

The use of molecular typing for characterization of blood group genes has been steadily increasing in the last few decades due to the transfusion benefits for the patients, technological advances in molecular techniques, and expanding availability of mass-scale genotyping. While blood group genotyping is becoming increasingly used, typing errors have been documented indicating the need for quality control.

International Workshops on Molecular Blood Group Genotyping have reported discrepancies between laboratories studying the same samples indicating that there is room for improvement [91, 92]. As a genotyping test may only be done once in a person's lifetime, errors can have serious consequences. A major challenge in performing routine RBC genotyping is controlling for process variability of molecular assays that arises due to scarcity of reference materials. This limitation has been circumvented by using clinical specimen leftovers from previous testing, which lack proper characterization. Reference materials are critical for the development and manufacture of testing kits, for test calibration and for monitoring of assay performance.

Recommendations on which targets and recommended controls to use for prediction of RBC antigens have been published by international societies offering proficiency test programs such as INSTAND [93], The Consortium for Blood Group Genes (CBGG) [94], College of American Pathologists (CAP), and International Society of Blood Transfusion (ISBT) [91, 92] and also by The American Association of Blood Banks (AABB) [95] whose focus is on creating guidelines for AABB accreditation aiming to certify laboratories that perform molecular testing for red cell, platelet, and neutrophil antigens.

Molecular testing laboratories participate in accreditation programs to validate their activities. In the US, the requirements for AABB accreditation are described in the 4th edition of standards for molecular testing for red cell, platelet, and neutrophil antigens (updated in October 2018) [95]. According to the document, "laboratories shall use appropriate reference DNA to validate and control the reported test" and further, "the reference DNA needs to contain the target polymorphisms reported by the laboratory". The same publication lists the blood group

alleles within 17 blood group systems that should be included in the reference DNA materials by the lab seeking to meet the minimum requirements for accreditation.

Various preparations have been used as controls to monitor performance of blood group genotyping assays, including synthetic DNA (such as PCR products, plasmid-cloned PCR products) and genetic material from human samples col¬lected and characterized for that purpose following standard guidelines [96]. PCR products and plasmid-cloned PCR products are simple and easy to produce and use as controls for SNPs, but they lack the genomic complexity of human sample and do not represent the clinical analyte (genomic DNA); moreover, the synthetic material can be a source of contamination for the laboratory if not carefully handled and well diluted. Although well-characterized human specimens are the best representation of clinical samples, they are of limited source and the replenishment may not come from the same donor. The alternative to overcome source limitation is to transform human cells into immortalized cell lines and use those for characterization and formulation of reference reagents. This approach has been successfully used to produce reference DNA using B-lymphoblastoid cell lines (B-LCLs), which are an appropriate representation of the genetic material of the donor [97].

B-LCLs can be generated by Epstein Barr Virus (EBV) infection of peripheral mononuclear cells from genetically characterized donors carrying specific blood group antigens and variants. The infection leads to proliferation and subsequent cell immortalization, providing an unlimited source of donor's genomic DNA. Once the B-LCLs are established, master and working cell banks are maintained in liquid nitrogen to ensure long-term survival of the cell line and continuous supply of the DNA. To produce the DNA reagent, B-LCLs from the working cell banks are expanded for bulk DNA extraction and subsequent DNA lyophilization. Tests on the lyophilized reagents are performed for assessment of their stability under accelerated degradation conditions and under normal conditions over longer periods of time as long-term stability. The material must be validated via collaborative studies with laboratories that routinely perform molecular blood typing.

The first DNA reference reagents for blood group genotyping developed using B-LCLs were produced in 2013 by the National Institute for Biological Standards and Control (NIBSC) and serve as World Health Organization (WHO) International Reference Reagents (IRR) for common blood group alleles found in ancestral Caucasians and Black African populations. The panel includes four DNA samples covering the most clinically important homozygous and heterozygous genotypes within RH, FY, KEL, JK, DO, and MNS blood group systems (**Table 4**). The material was validated in an international collaborative study by PCR-ASP or PCR-SSP, PCR-RFLP, Multiplex SSP assays, real-time PCR, Immucor Beadchip array, Progenika BLOODchip array, Luminex array, and 5′ Nuclease assay [98].

Additional cell lines were produced by CBER-FDA for use as source of genomic DNA for development of reference reagents to expand the number of red cell blood group polymorphisms represented in the first WHO IRR from 2013 for blood group genotyping. The CBER reference panel consists of 18 members, covering genotypes associated with 40 polymorphisms within 17 blood group systems, including alleles present in the existing WHO IRR-2013 except for *RHD*ψ* (**Table 4**). The CBER panel was also characterized and validated for additional genotypes belonging to systems already included in the WHO IRR-2013 (RH, KEL, FY, JK, DO, and MNS) (**Table 4**), and for additional systems only represented in CBER panel (ABO, LU, DI, YT, SC, CO, LW, CR, KN, IN, and OK) (**Table 5**) [89].

Participants in the international collaborative study to validate the CBER panel used traditional molecular techniques and additional genotyping techniques not available at the time of the first WHO IRR production. The most common methods

Blood group system	WHO IRR [98]	CBER RR [89]
Rh	*RHD* positive (zygosity not determined)	*RHD*01/RHD*01* (*RHD* homozygous)
		*RHD*01/RHD*01N.01* (*RHD* hemizygous)
	*RHD*01 N.01/RHD*01 N.01*	*RHD*01 N.01/RHD*01N.01* (homozygous *RHD* deletion)
	*RHD*01 N.01/RHD*ψ*	
	*RHCE*C/RHCE*c*	*RHCE*C/RHCE*c* (307T/C; 109 bp intron 2 ins present)
	*RHCE*C/RHCE*C*	*RHCE*C/RHCE*C* (307T/T; 109 bp intron 2 ins present)
	*RHCE*c/RHCE*c*	*RHCE*c/RHCE*c* (307C/C; 109 bp intron 2 ins absent)
	*RHCE*E/RHCE*e*	*RHCE*E/RHCE*e* (676C/G)
	*RHCE*e/RHCE*e*	*RHCE*e/RHCE*e* (676G/G)
		Additional polymorphisms:
		***RHCE* c. 122A>G (genotypes A/A; A/G)**
		***RHCE* c. 106G>A (genotype G/G)**
		***RHCE* c. 733C>G (genotype G/C; G/G; C/C)**
		***RHCE* c. 1006G>T (genotype G/G)**
Kell	*KEL*01/KEL*02*	*KEL*01/KEL*02* (578T/C)
	*KEL*01/KEL*01*	*KEL*01/KEL*01* (578T/T)
	*KEL*02/KEL*02*	*KEL*02/KEL*02* (578C/C)
		Additional polymorphisms:
		***KEL* c. 841C>T (genotypes C/C; C/T; T/T)**
		***KEL* c. 1790T>C (genotypes T/T; C/T; C/C)**
Duffy	*FY*01/FY*02*	*FY*01/FY*02* (125G/A)
		*FY*01/FY*01*(125G/G)
	*FY*02/FY*02*	*FY*02/FY*02* (125A/A)
	*FY*02N.01/FY*02N.01*	*FY*02N.01/FY*02N.01* (—67T/T)
		*FY*01/FY*02N.01* (—67T/C)
		Additional polymorphisms:
		***FY* c. 265C>T (genotypes C/C; C/T)**
Kidd	*JK*01/JK*02*	*JK*01/JK*02* (838G/A)
	*JK*01/JK*01*	*JK *01/JK*01* (838A/A)
	*JK*02/JK*02*	*JK *02/JK*02* (838G/G)
Dombrock		*DO*01/DO*02* (793A/G)
		*DO*01/DO*01* (793A/A)
	*DO*02/DO*02*	*DO*02/DO*02* (793G/G)
		Additional polymorphisms:
		***DO* c. 323G>T (genotypes G/G; G/T)**
		***DO* c. 350C>T (genotype C/C; C/T; T/T)**

Blood group system	WHO IRR [98]	CBER RR [89]
MNS	*GYPA*M/GYPA*M*	*GYPA*M/GYPA*M* (59C/C)
	*GYPA*M/GYPA*N*	*GYPA*M/GYPA*N* (59C/T)
	*GYPA*N/GYPA*N*	*GYPA*N/GYPA*N* (59 T/T)
		*GYPB*S/GYPB*S* (143 T/T)
	*GYPB*S/GYPB*s*	*GYPB*S/GYPB**s (143C/T)
	*GYPB*s/GYPB*s*	*GYPB*s/GYPB*s* (143C/C)
		Additional polymorphisms:
		***GYPB* c. 230C>T (genotype C/C)**
		***GYPB* c. 270+5G>T (genotype G/G)**

Notes: Additional polymorphisms and genotypes included in the CBER panel are in bold; in parentheses are genotypes represented in the CBER panel members; descriptions of the polymorphism positions/genotypes are not available for WHO panel. For additional details see references [89, 98].

Table 4.
Alleles or genotypes included in the WHO IRR and CBER RR for the overlapping blood group systems.

Blood group system	Gene	Polymorphism	Genotypes included
ABO	*ABO*	c.1061delC	CC; C/delC
		c.526C>G	C/C; G/C
		c.703G>A	G/G; G/A
		c.796C>A	C/C; C/A
		c.803G>C	G/G; G/C
		c.261delG	G/G; G/delG, delG/delG
Lutheran	*BCAM*	c.230G>A	A/A; G/A; G/G
Diego	*SLC4A1*	c.2561C>T	C/T; C/C
Cartwright	*ACHE*	c.1057C>A	C/C; C/A; A/A
Scianna	*ERMAP*	c.169G>A	G/G; G/A
Colton	*AQP1*	c.134C>T	C/C; C/T; T/T
Landsteiner-Wiener	*ICAM4*	c.299A>G	A/A; A/G
Cromer	*CD55*	c.679G>C	G/G; G/C
Knops	*CR1*	c.4681G>A	G/G; G/A
		c.4768A>G	A/A; A/G
		c.4801A>G	A/A; A/G; G/G
Indian	*CD44*	c.137G>C	G/G
OK	*BSG*	c.274G>A	G/G

For additional details, see reference [89].

Table 5.
Genotypes from additional blood group systems covered by the CBER panel members.

used by the collaborators were PCR-SSP, either single- or multiplex, the HEA (human erythrocyte antigen), RHD, and RHCE BeadChip arrays from Immucor, Sanger sequencing, PCR-RFLP, and real-time PCR based assays. Less common methods included ID-CORE XT and BLOODchip REFERENCE by Progenika, MALDI-TOF-based assays such as Hemo ID from Agena Bioscience, NGS, RBC-Ready Gene and

RBC-FluoGene by Inno-train Diagnostik GmbH, droplet digital PCR, HI-FI Blood by AXO Science, SNaPshot, and high-resolution melting analysis (HRMA) [89].

6. Conclusions

Alloimmunization remains a major risk for transfusion-dependent patients for whom transfusion is critical for survival. The transfusion management of patients who have already been alloimmunized is still a challenge when rare blood types are involved, but for new patients there is a hope that genotyping will help minimize exposure, except for those very rare alleles which are hard to find. One approach to overcome this limitation is to create a database for rare and very rare RBC alleles where donor selection is based on genotyping and the donor pool is constantly enhanced and updated. The use of RBC genotyping for both rare donor selection and patient care is path forward in transfusion therapy and transfusion safety. The development of new assays and high throughput platforms will enable mass-scale genotyping at lower cost and rapid pace to select rare and very rare donors. The development and availability of reference reagents will allow better quality control in assay development and evaluation of performance and proficiency of testing by specialized laboratories making patient care easier and safer to provide.

Conflict of interest

The authors have no conflict of interest.

Disclaimer

The content of this chapter represents the authors' opinion and does not represent FDA judgment; therefore, it does not bind or obligate the FDA.

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