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Review Article

Significant of Molecular Genotyping over Serological Phenotyping Techniques in Determination of Blood Group Systems among Multiple Transfused Patients and Blood Donors to Prevent Alloimmunization

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Summary

Erythrocyte serological phenotyping is very important in determining the identity of suspected alloantibodies and also to facilitate the identification of antibodies that may be formed in the future. Serological phenotyping is a conventional method which is based on the presence of visible haemagglutination or haemolysis. This technique has some limitations in successful determination of blood group due to presence of donor red blood cell in the circulation of recent multiple transfused patients, taking certain medications or some diseases condition which may alter the erythrocyte composition, this make accurate determination of blood group of such patients to be time consuming and difficult to interpret. It is often more complicated to determine the blood group, if direct antiglobulin test of such patients were positive and there is no direct agglutinating antibody. Molecular Genotyping of blood group systems led to the understanding of the molecular basis of many blood group antigens, many blood group polymorphisms are associated with a single point mutation in the gene encoding of protein carrying the blood group antigen. This knowledge allows the use of molecular testing to predict the blood group antigen profile of an individual and to overcome the limitations of conventional serological blood group phenotyping. Determination of blood group polymorphism at the genomic level facilitates the resolution of clinical problems that cannot be addressed by serological techniques. Applications of blood group genotyping for red cell blood group antigens affecting several areas of medicine which includes identification of fetuses at risk for haemolytic disease of the newborn and candidates for Rh-immune-globulin, to determine antigen types for which currently available antibodies are weakly reactive, to determine blood group of patients who had recent multiple transfusion, to increase the reliability of repositories of antigen negative RBCs for transfusion, to select appropriate donor for bone marrow transplantation, to provide transfusion support for highly alloimmunized patients, to resolve ABO and Rh discrepancies, to confirm sub group of A2 status of kidney donors, to provide comprehensive typing for patients with haematological diseases requiring chronic transfusion and oncology patients receiving monoclonal antibody therapies that interfere with pretransfusion testing.

INTRODUCTION

Blood group systems are characterized by the presence or absence of antigens on the surface of erythrocytes, the specificity of antigens is controlled by a series of genes which can be allelic or linked very closely on the same chromosome which persist throughout life and serve as identity markers. Presently, International Society of Blood Transfusion (ISBT) has acknowledged about 36 blood group systems and more than 420 blood group antigens have been discovered on the surface of the human red cell [1]. The clinical importance of RBC antigens is associated with their ability to induce alloantibodies that are capable of reacting at 37^0C (body temperature), these antibodies have ability to cause destruction of erythrocytes. The major clinically significant antibodies include ABO, Rh, Kell, Kidd and Duffy antigens [2]. ABO antibodies are naturally occurring antibodies, while Rh and Kell antibodies are immunogenic in nature. Immune system produces alloantibodies when it is exposed to foreign antigens (incompatible erythrocyte) these antibodies form a complex with donor cells causing haemolytic transfusion reactions. Patients with Rh and Kell alloantibodies should be transfused with blood that is lacking the antigens because these antibodies are capable of causing severe haemolytic anaemia and haemolytic disease of the newborns [3]. Hence, it is important to

transfused females of child-bearing age with compatible blood in order to reduce or minimize the possibility of sensitizing their immune system with clinically important antigens [4]. Unexpected incompatibility reactions are the major risks of blood and blood products transfusion apart from transfusion transmissible infections, clinically significant alloantibodies play a critical role in transfusion medicine by causing either acute or delayed haemolytic transfusion reactions (HTRs) and haemolytic disease of the fetus and newborn (HDFN) ranging from mild to severe grades. The degree of production of alloantibodies that is capable of destroys foreign or donor red cells are higher amongst multi-transfused patients compared with general population. Serological phenotyping of blood group system is a classical and conventional method of detecting erythrocyte antigen by haemagglutination or haemolysis, accurate phenotyping of blood group system among multi-transfused patients is a very complex process due to the presence of donor's blood cells in the patient's circulation except serological phenotyping of blood group system is performed before the initiation of transfusion. Blood group genotyping has recently been developed to determine the blood group antigen profile of an individual, with the goal of reducing risk or identify a fetus at the risk of haemolytic disease of the newborn (HDN). Blood group genotyping improve the accuracy of blood typing where serology alone is unable to resolve red cell serological phenotyping especially in individuals with weak antigen expression due to presence of genetic variants or in case of rare phenotype where antisera are unavailable or in the case of recent multiple blood or blood products transfusion, or in patients whose RBCs are coated with immunoglobulin [5]. Genotyping technique also help to determine which phenotypically antigen-negative patients can receive antigen-positive RBCs, to type donors for antibody identification panels, to type patients who have an antigen that is expressed weakly on RBCs, to determine Rh D zygosity, to mass screen for antigen-negative donors, ability to routinely select donor units antigen matched to recipients apart from ABO and Rh D which reduce complications in blood and blood products transfusion [6]. The growth of whole-genome sequencing in chronic disease and for general health will provide patients' comprehensive extended blood group profile as part of their medical record to be used to inform selection of the optimal transfusion therapy [7]. DNA-based genotyping is being used as an alternative to serological antibody-based methods to determine blood groups for matching donor to recipient because most antigenic polymorphisms are due to single nucleotide polymorphism changes in the respective genes. Importantly, the ability to test for antigens by genetic technique where there are no serologic reagents is a major medical advance and breakthrough to identify antibodies and find compatible donor units, which can be life-saving. The molecular genotyping of blood group antigens is an important aspect and is being introduced successfully in transfusion medicine. Genotyping has been shown to be effective and advantageous in relation to phenotype from genomic DNA with a high degree of precision [8] A notable advantage of molecular testing is its ability to identify variant alleles associated with antigens that are expressed weakly or have missing or altered epitopes, thus helping to resolve discrepant or incomplete blood group phenotyping. The disadvantages of molecular testing are mainly the longer turnaround time and higher cost, compared with serologic typing [9]. The molecular basis for most erythrocyte antigens is known and numerous DNA analysis methodologies have been developed, all based on PCR which can detect several alleles simultaneously as long as the alleles studies have products of different sizes [10]. The detection of blood group antigens is essential in transfusion practice in order to prevent alloimmunization, especially in multiple transfused patients. Erythrocyte antibodies that are clinically significant in transfusion medicine can lead to acute or delay blood transfusion reactions and haemolytic disease of the fetus and newborn which increases the morbidity and mortality rate of the patients. In addition, alloimmunization may delay the localization of a compatible blood bag. The probability of an individual producing one or more anti-erythrocyte antibodies is approximately 1% per unit of blood transfused and in chronically multiple transfused patients, the alloimmunization rate may reach 50%. Both the blood donors and recipients can be genetically typed for all the clinically significant blood group antigens and antigen-matched blood can be provided to the recipient [6]. This approach could significantly reduce the rate of alloimmunization.

Serological phenotyping

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, organ transplantation, cancer treatment, haematological diseases (like sickle cell disease, thalassaemia, and aplastic anemia). Serology has been considered the gold standard technique for blood group typing for a long time [11]. 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 [12]. Scarcity of serological reagents for some blood group systems for which there is no monoclonal antibody available is a major limitation to serological technique. 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.

Molecular Genotyping

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 resolution in complex cases and overcome limitations of serological techniques

when dealing with allo-immunized and multi-transfused patients [8]. In addition, molecular techniques have allowed identification of genes encoding clinically relevant antigens where serological reagents are not available. 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 [7]. 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 (for example ABO, RH, and DO blood group systems), sequence duplication, (for example RHD gene and GE blood group system), nonsense mutation (for example RHD gene), and hybrid genes (for example RH, MNS, ABO, and CH/RG blood group systems) [13]. In contrast to serological technique, molecular genotyping 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 and blood products transfusions. Thus, erythrocyte genotyping can resolve blood group typing discrepancies in multi-transfused patients presenting with mixed field reactions, alloantibodies, or autoantibodies. 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 [4]. Studies comparing serology and genotyping in multi-transfused population such as patients with thalassaemia and sickle cell disease have shown that genotyping is superior to serology for resolving discrepancies. Use of genotyped matched units has been shown to decrease alloimmunization rates, increase haemoglobin levels and in vivo erythrocyte survival, and diminish frequency of transfusions.

Erythrocyte Antigen Disparity and its significant in Transfusion Medicine

Patients who develop alloantibodies might have been received significantly multiple transfusions, or due to pregnancy making alloimmunization a particular problem for such patients especially those requiring chronic RBC transfusion support as a result of haematological diseases [14]. Incidence of alloimmunization is highly variable between individual patients' health condition, rate of exposure to a foreign antigen, ethnicity and geographical area. However, Knowledge of the genotypes of both patients and donors has led to a greater understanding of potential mechanisms for persistent alloimmunization despite serologic antigen matching for transfusion, also extended matching to include the Duffy, Kidd, and MNS systems has been shown to reduce the rate of alloimmunization [15]. It is therefore clear that serologic phenotyping is inadequate to capture allelic diversity in minority populations. Without accurate characterization of the patient and donor genotypes, true antigen matching to prevent alloimmunization is not possible [16]. In addition, there is still an inadequate understanding of the risk of alloimmunization with specific blood group gene haplotypes, particularly for *RHD*. Large, multi-institutional studies with genotyping of both patient and donor and better characterization of the specificity of antibodies formed are needed to clarify the clinical significance and immunogenic risks of variant alleles [17].

Blood transfusion and risk of erythrocyte alloimmunization

Erythrocyte alloimmunization is a serious adverse event of blood and blood products transfusions which can cause further clinical problems in the recipient patients including worsening of anaemia, development of autoantibodies, acute or delayed haemolytic transfusion reactions, bystander haemolysis, organ failure, and cause serious complications during pregnancies [3]. 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 [11]. Reported erythrocyte alloimmunization rates have considerable variations depending on the population and disease studied. 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 sickle cell disease, rates vary between 8 and 76% [16]. 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 [18]. Studies in sickle cell disease 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. Several studies have suggested that genetic variation in immune-related genes and human leukocyte antigens might be associated with susceptibility to or protection from alloimmunization [19].

Consequences of Alloimmunization in transfusion medicine

Depending on the antigen and clinical significance of the antibody formed, patients can suffer morbidity and mortality due to an acute or delayed hemolytic transfusion reaction if incompatible blood or blood products are transfused [15]. A rare but life-threatening consequence of recurrent transfusions is a hyperhaemolytic reaction which occurs in patients with hemoglobinopathies especially in sickle cell disease patients, the mechanism of hyperhaemolytic reaction in SCD could be a complication of alloimmunization, with possible contribution of an underlying genetic predisposition [17]. The development of RBC alloantibodies also impacts patient care by increasing the cost and time required to find compatible RBC units. Once an RBC antibody is identified, all subsequent transfusions must be negative for that antigen to prevent a delay haemolytic transfusion reaction from a robust secondary immune response [14]. An additional risk for previously sensitized patients is the inability to detect evanesced RBC antibodies at future transfusion events, failure to identify pre-existing antibodies is a significant contributor to haemolytic transfusion reaction.

Minority patients may be at greater risk of complications from alloimmunization because the presence of antibodies may not be accurately characterized. One reason is that they are more likely to be negative for high-prevalence antigens [15]. Antibodies to high-prevalence RBC antigens will react with all reagent RBCs. This is further complicated if the patient also has a positive direct antiglobulin test (DAT), as patients with SCD frequently do; the antibody to a high-prevalence antigen can then easily be confused with a warm autoantibody **[20]**. In addition, because most reagent RBCs are not from minority populations, there is a risk that immunogenic Rh variants and other low-prevalence antigens are not expressed on the reagent RBCs, rendering antibody detection tests false-negative. Genotyping can be particularly useful to clarify antibody specificity, identify if there is a lack of a high-prevalence antigen, and identify appropriate donors.

Prevention of alloimmunization and improvement of transfusion therapy

Prevention of alloimmunization is desirable for any blood and blood products transfusion. Hence, 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 [21]. Patient's previously transfused, particularly transfusion-dependent patients, the alloimmunization risk are 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 [3]. 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 to prevent alloimmunization [10]. 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 haemolytic 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 delay haemolytic transfusion reaction and haemolytic disease of foetus and newborn [16]. Currently, recommendations for partial and extended donor unit or patient matching are limited to specific groups including patients on long-term transfusion protocol (sickle cell disease, thalassaemia, and aplastic anemia), patients who have developed alloantibodies and patients with warm autoimmune haemolytic anaemia [22]. 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 (Fya, Fyb), JK (Jka, Jkb), MNS (S, s) and, if available, additional antigens [23]. Prevention of an initial alloimmunization event may be even more important than previously appreciated to prevent the development of subsequent antibodies. For patients with a tendency toward forming RBC antibodies, and also having a RBC phenotype with either multiple negative antigens and/or lacking high-prevalence antigens, compatible units may become so rare as to make transfusion support virtually impossible [24].

Screening for clinically significant alloantibodies

Alloantibodies are antibodies produced in a patient as a result of exposure to foreign red cell antigen through transfusion of blood or blood products, pregnancy or transplantation [21]. In countries such as Nigeria, there are multiple ethnic groups and racial or genetic heterogeneity among the population. This can often be associated with a wide variation of alloantibodies. Other common factors that facilitate alloantibody formation in the recipient include: the immune competence, the dose of the antigen the recipient is exposed to, the route of exposure and how immunogenic the foreign antigen is [25]. Development of alloantibodies can lead to difficulty in finding compatible blood for transfusion or it can result in severe delayed haemolytic transfusion reaction if the antibody titre is low, undetected, missed and if antigen positive units is transfused. Evidenced-based best practice in the developing world requires that alloantibody testing is carried out as part of pre-transfusion testing of patients who require a red cell transfusion as well as pregnant women presenting to antenatal clinic at booking [4]. The purpose of this test is to detect the presence of unexpected red cell antibody in the patient's serum. Once these antibodies are detected during the alloantibody screening, every effort must be made to identify the specificity of the alloantibody by doing a panel test. The aim of identifying the specificity of the alloantibody in a patient that requires a red cell transfusion is to enable the Medical Laboratory or Biomedical Scientist to select antigen negative donor unit for appropriate crossmatch (indirect ant globulin test) for such patient [21]. Panel test in the case of a pregnant women coming for antenatal booking is to identify the alloantibody, determine whether the antibody can potentially cause HDFN and to allow the monitoring of the titre or quantification of the antibody every 4 weeks from booking until 28 weeks' gestation and every 2 weeks thereafter until delivery. This information is important to determine the extent of developing foetus is affected by HDFN, decide whether to monitor the baby for anaemia using Doppler ultrasound, determine whether the baby will require intrauterine transfusion and to make an informed decision to possibly deliver the baby earlier. These evidence-based best practices are not being implemented in many settings in Nigeria [25]. Testing of donor units for other clinically relevant red cell antigens other than ABO and Rh D is not routinely carried out [3]. This is a complete failure in stewardship by the Nigerian government and can compromise the transfusion service delivery to pregnant women and patients that require red cell transfusion. Settings and implement a policy to routinely test all group O donor units for haemolysins in other to identify group O donors with high titre of IgG anti A and/or anti B whose blood should be reserved only for transfusion to group O recipient while those that test negative can be transfused to A, B or AB individual as a way to maximizing the use of our limited allogeneic stock [26].

Applications of molecular genotyping over serological phenotyping in transfusion medicine

Multiply-transfused patients: The ability to determine a patient antigen profile by DNA analysis when haemagglutination tests cannot be used is a useful adjunct to a serologic investigation. Blood group genotyping in the transfusion setting is recommended for multiply transfused patients such as sickle cell disease (SCD), as part of antibody identification process [27]. Determination of a patient's blood type by analysis of DNA is particularly useful when a patient, who is transfusion-dependent, has produced alloantibodies, this will help in the selection of antigen-negative RBCs for transfusion. It also assists in selection of compatible units for patients with discrepancies between genotype and phenotype, leading to increased cell survival and a reduction of the transfusion frequency [13]. In addition to its contribution to the general accuracy of identification of red blood cell antigens, genotyping of transfusion-dependent SCD patients allows assessment of the risk of alloimmunization against antigens

Patients whose RBCs are coated with IgG: Patients with autoimmune haemolytic anaemia (AIHA), whose RBCs are coated with IgG cannot be accurately typed for RBC antigens, particularly when directly agglutinating antibodies are not available, or IgG removal by chemical treatment of RBCs is insufficient. Blood group genotyping is very important for determination of the true blood group antigens of these patients [20]. Patients received antigen-matched RBCs typed by blood group genotyping increases erythrocytes in vivo survival, as assessed by rises in haemoglobin levels and diminished frequency of transfusions.

Blood donors: DNA-based typing can also be used to antigen-type blood donors both for transfusion and for antibody identification reagent panels. This is particularly useful when antibodies are not available or are weakly reactive [28]. The molecular analysis of a variant gene can also assist in resolving a serologic investigation.

Resolution of weak A, B, and D typing discrepancies: A proportion of blood donors and patients who historically have been typed as group O are now being recognized as group A or group B with the use of monoclonal antibodies capable of detecting small amounts of the immuno-dominant carbohydrate responsible for A or B specificity [12]. A typing result that differs from the historical record often results in time-consuming analyses. Since the bases of many of the weak subgroups of A and B are associated with altered transferase genes, PCR-based assays can be used to define the transferase gene and thus the ABO group [29]. Similarly with the D antigen of the Rh blood group system, a proportion of blood donors that historically have been typed as D-negative are now reclassified as D-positive, due to monoclonal reagents that detect small and specific parts of the D antigen. The molecular basis of numerous D variants can be used to identify the genes encoding altered Rh D protein in these individuals [28].

Applications to maternal-fetal medicine

Alloimmunization against the Rh D antigen during pregnancy is the most frequent cause of haemolytic disease of the newborn (HDN). Immunization occurs when fetal cells, carrying antigens inherited from the father, enter the mother's circulation following fetal-maternal bleeding. The mother, when not expressing the same antigen(s), may produce IgG antibodies towards the fetal antigen and these antibodies can pass through the placenta causing a diversity of symptoms, ranging from mild anaemia to death of the foetus [25]. Apart from antibodies to the Rh D blood group antigen, other specificities within the Rh system and several other blood group antigens can give rise to HDN, but Rh D is by far the most immunogenic. Prenatal determination of fetal Rh D status is desirable in pregnancies to prevent sensitization and possible hydrops foetalis in foetuses of Rh D negative mothers with Rh D positive fathers. Fetal DNA has been detected in amniotic cells, chorionic villus samples, and as recently reported, in maternal plasma. It is now well accepted that a minute number of copies (as low as 35 copies/mL) of cell-free fetal *RHD* DNA in the maternal plasma can be utilized as a target for non-invasive genotyping of the foetus [10]. Unlike fetal DNA isolated from the cellular fraction of maternal blood samples, free fetal DNA isolated from maternal plasma has been shown to be specific for the current foetus and is completely cleared from the mother's circulation by postpartum. It has been reported that fetal *RHD* can be determined by PCR in DNA extracted from maternal plasma of pregnant women with Rh D positive foetuses, in a non-invasive procedure. PCR amplification of *RHD* in maternal plasma may be useful for the management of Rh D negative mothers of Rh D positive foetuses and for the study of foetus-maternal cell trafficking [30].

CONCLUSIONS

Determination of blood group polymorphism at the genomic level facilitates the resolution of clinical problems that cannot be addressed by serological technique. They are useful to determine antigen types for which currently available antibodies are weakly reactive; to type patients who have been recently transfused; to identify fetuses at risk for haemolytic disease of the newborn and to increase the reliability of repositories of antigen negative RBCs for transfusion. Mass scale genotyping, if applied to routine blood group of patients and blood donors, would significantly change the management of blood provision. Better matching of donor blood to patient would be the most significant benefit. This is primarily because a large numbers of low frequency antigens (or absence of high frequency antigen) are not routinely tested for, and donor-patient mismatches are only detected by serological cross-matching (only if an antibody has been generated) immediately prior to transfusion. This review overviews the current situation in this area and attempts to predict how blood group genotyping will evolve in the future.

LIMITATION

It is important to note that PCR based assays are prone to different types of errors than those observed with serological

assays. For instance, contamination with amplified products may lead to false positive test results. In addition, the identification of a particular genotype does not necessarily mean that the antigen will be expressed on the RBC membrane.

RECOMMENDATION

As a word of caution, we should emphasize that the interpretation of molecular blood group genotyping results must take into account that potential contamination of PCR-based amplification assays and the observation that the presence of a particular genotype antigen does not guarantee expression of this antigen on the RBC membrane. The possibility to have an alternative to serological tests to determine the patient's antigen profile should be considered for multiply transfused patients and for patients with autoimmune haemolytic anaemia (AIHA) by allowing the determination of the true blood group genotype and by assisting in the identification of suspected alloantibodies and in selection of antigen-negative RBCs for transfusion. This ensures a more accurate selection of compatible donor units and is likely to prevent alloimmunization and reduce the potential for haemolytic reactions. As automated procedures attain higher and faster throughput at lower cost, blood group genotyping is likely to become more widespread. We believe that the PCR technology may be used in a transfusion service in the next few years to overcome the limitations of serological technique.

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