Importance of Extended Blood Group Genotyping in Multiply Transfused Patients

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Abstract

Blood group antigen systems are not limited to the ABO blood groups. There is increasing interest in the detection of extended blood group systems on the red cell surface. The conventional method used to determine extended blood group antigens or red cell phenotype is by serological testing, which is based on the detection of visible haemagglutination or the presence of haemolysis. The method relies on the use of monoclonal or polyclonal antibodies designed to detect specific epitopes of the antigens on the red cell surface. However, this technique has many limitations. The replacement of the conventional serologic method is needed in cases where blood group antigen detection by this method is not reliable. This may be due to recent exposure to donor red cell, certain drugs or medications or other diseases that may alter the red cell membrane. The main aim of this study is to determine the red cell blood group genotype by PCR and to compare the results with the conventional serological methods in multiply transfused patients. Sixty-three patients participated in this study. Peripheral blood was collected and blood group phenotype was determined by serological tube method while the genotype was performed using TaqMan Single Nucleotide Polymorphism (SNP) RT-PCR assays for RHEe, RHCc, Kidd and Duffy blood group systems. Discrepancies were found between the phenotype and genotype results for all blood groups tested. Accurate red blood cell antigen profiling is important for patients requiring multiple transfusions. The SNP RT-PCR platform is a reliable alternative to the conventional method.

Keywords Blood group, genotyping, multiply transfusion, red cell

1. Introduction

Transfusion management amongst multiply transfused patients are often complicated. The rates of alloimmunization or the formations of antibodies that may potentially destroy foreign or donor red cells amongst multi-transfused individuals are significantly higher compared to the general population. The formation of clinically significant Red Blood Cell (RBC) alloantibodies can cause major problems to the recipient. However, accurate phenotyping of RBC from this group of patients is very complex process due to the presence of donor's blood cells in the patient's blood circulation (Reid & Yazdanbakhsh, 1998) unless the phenotyping is performed prior the initiation of transfusion.

Blood group antigen systems are not limited to the A, B, AB and O blood groups only. To date, The International Society of Blood Transfusion (ISBT) has acknowledged 33 blood group systems with more than 300 blood group antigens described on the surface of the human red cell (Anstee, 2009; Transfusion, 2014). However, amongst all the blood group systems, the clinically significant groups that have been identified are mainly RH (D, Cc, Ee), Kidd (Jk a, Jk b), Duffy (Fy a, Fy b). The antibodies formed from these blood group systems causes significant red cell destruction. Supplying the accurate phenotyping of blood group antigen is necessary to prevent alloimmunization from occurring in susceptible patients.

Determination of blood group antigens is performed by serological test, which is regarded as the gold standard method for blood group typing by using the specific antisera to detect the specific antigens on the red blood cells surface. Nevertheless, this technique has many limitations especially for the repeated transfusion patients where the results may not be reliable (Lilian Castilho et al., 2002; L. Castilho, Rios, Pellegrino, S, & F, 2002; Guelsin et al., 2010; Reid ME, Rios M, Powell D, Charles-Pierre D, & V, 2000; Ribeiro et al., 2009; Rožman, Dovč, & Gassner, 2000). In recent years, several molecular methods for RBC typing have been used to resolve the problems with haemagglutination test. As the molecular basis of almost all blood group antigens has been determined (Daniels, 2005), it is now possible to predict the blood group antigen profile of an individual by testing their DNA with a high degree of accuracy (J. M. Moulds, 2010; Westhoff, 2006). Because of the genetic code of an individual is contained in the DNA in all somatic cells, it is possible to perform DNA analysis from any source even though there is an issue where there is a presence of donor DNA in recipient blood (Adams PT, Davenport RD, Reardon DA, & MS, 1992; Carter AS, Bunce M, & Cerundolo L, 1998; Lee TH, Paglieroni T, & Ohto H, 1999; Lee, Donegan, Slichter, & Busch, 1995). However, determining the gene or allele that is relevant and prevalent in a particular population is also a challenge. Little is documented on the extended blood group genotypes in transfusion-dependent patients in Malaysia.

An alternative to this method is needed to provide the accurate extended blood antigen typing in these patients. Therefore, in the current study, we compared the phenotype and genotype results from multiply transfused patients. The blood phenotyping was determined by haemagglutination test while the blood genotyping was performed using TaqMan SNP RT-PCR assays by using DNA preparation from the same blood sample.

2. Body of Paper

2.1 Methodology

2.1.1 Study subjects

Sixty-three multiply transfused patients aged 18 years old and above, receiving treatment and follow up at the Thalassaemia Clinic, Hospital Ampang Selangor and Universiti Kebangsaan Malaysia Medical Centre (UKMMC) agreed to participate in this study by signing an informed consent form. All of these patients had received 2 or more units of donor RBC previously and had received transfusion within the previous 3 to 12 weeks.

2.1.2 Control group

Red cell phenotyping and genotyping studies were performed on 20 healthy volunteers who participated in the study by signing the informed consent form.

2.1.3 Samples used for analysis

Peripheral blood was collected in two separate K2EDTA tubes of 3.5 ml. One tube was used for serological red cell phenotype and the other tube was used for DNA extraction from the buffy coat layer of nucleated cells.

2.1.4 Serology test to determine red blood cell phenotype

The phenotype of the patients' red blood cells was performed by using tube method. Specific antisera was used against 3-5% of patients' red cells in normal saline suspension. This was performed for RH (C, c, E, e), Kidd (Jk a, Jk b) and Duffy (Fy a, Fy b) blood group system according to standard serologic manufacturer's protocols (DiaMed GmbH 1785 Cressier FR, Switzerland). Visible haemagglutination was graded. Presence of haemolyis was regarded as positive. A mixed-field reaction was considered indeterminate. The test reaction results were recorded according to the accepted international protocols in American Association of Blood Banks (AABB) technical manual.

2.1.5 DNA extraction

Two hundred μl of packed red cells was pipetted into a sterile 1.5 ml micro centrifuge tube. DNA was extracted using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen, Germany). The protocol was followed with minor modifications. At the elution step, 30 μl of AE buffer was added instead of 200 μl and the incubation time at room temperature was increased to 10 min. The elution step was repeated twice. The genomic DNA was quantified using NanoPhotometer® P-Class analysis and was stored at -20°C until used for molecular DNA genotyping.

2.1.6 SNP RT-PCR analysis

Molecular DNA genotyping was performed using Single Nucleotide Polymorphism (SNP) RT-PCR. Oligonucleotide primers used were obtained either as predesigned or custom-designed based on locus files submitted to Applied Biosystems for RHEe, RHCc, Kidd and Duffy system (rs609320, rs676785, rs1058936 and rs12075, respectively). Each respective allele was tagged with a specific fluorescent signal. The protocols were carried out based on the manufacturer's instructions according to the standard 10-μL reaction volume TaqMan assay protocol by using Applied Biosystems 7500 Fast Real Time PCR Systems V2.0.6 (Applied Biosystems, USA). PCR mixture containing 10 ng templates of DNA was used in the reaction. Results were viewed in the TaqMan Genotyper Software V1.0.3 as individual data points for each reaction on the Cartesian plot representing the signal intensity of the fluorescent VIC reporter (allele one) versus signal intensity of the fluorescent FAM reporter (allele two). Genotype calls were determined by interpretation of the ratio of VIC signal to FAM signal for each system. Reaction clusters obtained at the x/y axis that do not contain the template of DNA were used as negative controls for the experiment.

2.1.7 Ethical Consideration

This study was approved by the Medical Research Ethical Committee of the Ministry of Health, Malaysia and registered with the National Medical Research Register (Research ID 12-567-12622)

and Universiti Kebangsaan Malaysia Medical Centre (Research ID FF-419-2012). This study also was funded by the Ministry of Higher Education from the Exploratory Research Grant Scheme (ERGS/1/2012/SKK06/USIM/03/1).

2.2 Results

2.2.1 Types of multiply-transfused cases

Patients consisted of 24 males and 39 females who were transfusion-dependent individuals aged range between 18-65 years old (median age of 28 years old). The types of patients are shown in Table 1. Amongst the thalassaemia cases, thalassaemia intermedia represented the highest number of patients. Patients received a blood transfusion as frequent as every 4 weekly intervals, shown. There are 3 types of donor red blood cell product that patients received during transfusion; filtered red blood cells (FRBC), packed red blood cells (PC) and buffy-coat poor packed cells (BCPPC). A majority of patients received FRBC products.

2.2.2 Phenotype-genotype frequencies

All the patients were RHD positive. The phenotype frequencies determined serologically for RH, Kidd and Duffy have been shown in Table 2. The most frequent phenotype of the RH system were Rh C+c- and Rh E-e+, for Kidd system was Jk(a+b+) and for Duffy system was Fy(a+b+). However, there were a number of samples that were considered 'Undetermined' due to mixed-field reactions observed in all blood group systems tested (Table 2). We tested the patients' DNA using SNP RT-PCR. All of the patients' samples red cell genotypes were able to be determined (Table 2). The most frequent genotype of the RH system were, RHCE*CC (66.7%) and RHCE*ee (50%). The Kidd system was JK*A/JK*B (28%) and Duffy system was FY*A/FY*A (49%).

2.2.3 Correlation between phenotype and genotype results

Duffy blood group system showed the highest number of discrepancies between the red cell phenotype determined serologically and genotype determined using molecular techniques. Forty-seven cases were discordant (Table 3). The main discrepancy was found in FY*A/FY*A allele when serologically showed Fy(a+b+). The Kidd blood group system showed 41 discrepancies between the 2 methods used. Twenty-eight patients were found to have discrepancies between the serological-phenotype and genotype in the RH blood group system. Agreement between phenotype and genotype was observed for RH blood group system in 35 patients. Twenty-five patients are CCee/R1R1, 7 patients are CcEe/R1R2 and 3 patients are Ccee/R1r. There was full agreement between phenotype and genotype of the 20 healthy volunteers.

2.3 Discussion

The constant presence of donor RBC in the recipients' blood circulation renders the serological result invalid and in some instances inaccurate even when the test is performed correctly. Serological testing which is based on the detection of haemagglutination or haemolysis is the conventional method to determine the blood group antigens. Majority of hospital blood banks in

Malaysia are currently using this conventional RBC serology method to determine a probable or most likely genotype based on the expression of antigens on the red cell surface. Other than that, extended phenotype of blood group antigens is not performed on a routine basis, especially to patients that require repeated red cell transfusions to survive.

Most of our patients are transfusion-dependent thalassaemia patients that require a blood transfusion every 4 weeks. Based on the phenotype-genotype results, we have shown that there is mistyping when haemagglutination is performed that could lead to false assignment of blood group systems. Undetermined blood group systems were also detectable when mixed-field reactions were recorded. In the present work, 47 cases in Duffy system, 41 cases in Kidd system and 28 cases were found to have discrepancies in the RH blood group system. Undetermined phenotypes detected using the haemagglutination technique was resolved when the patient's DNA was subjected to red cell blood group system genotyping using the SNP RT-PCR. Mixed-field reaction denotes a mixture of more than 1 population of red cells and therefore a positive and negative reaction are both seen in the reaction tube. The most common allele detected for extended RH phenotype was RHCE*CC and RHCE*ee assigned as R1R1 (CDe/CDe), JK*A/JK*B assigned as Jk(a+b+) for Kidd and FY*A/FY*A assigned as Fy(a+b-) for Duffy (Table 3).

Previous studies have been reported that the high failure rate of serological antigen typing in multiply transfused patients makes the serological results unreliable (Lilian Castilho, et al., 2002; L. Castilho, et al., 2002; Guelsin, et al., 2010; Reid ME, et al., 2000; Ribeiro, et al., 2009; Rožman, et al., 2000). The phenotyping results have shown discordant with the genotyping results. In these studies, different types of molecular methods were employed amongst the multiply transfused patients with various haematological diseases and patients with renal failure. Mixed-field agglutination also was observed in more than one antigen typing which makes difficulties in interpreting of the patients' blood phenotype and determination of the antigen-matched RBCs for the patients. The proper selection of antigen-negative RBCs for regular transfusion patients can minimize the risk of alloimmunization as some of these blood group antigens can stimulate clinical significance antibodies and cause transfusion reaction. The blood group antigens that are usually implicated are Rhesus, Kell, Kidd and Duffy (Higgins & Sloan, 2008; Klein & Anstee, 2006; Transfusion, 2014). Once alloantibodies were developed, the management of thalassaemia patients will become more complicated as the antibodies will limits the availability as well as the safety of subsequent RBC transfusion (George, 2013). Vichinsky (Vichinsky, 2001) showed a reduction in alloimmunization rates among Sickle Cell Anaemia patients from 3% to 0.5% with transfusion of phenotypically matched blood while Singer and colleagues (Singer et al., 2000) showed highest rate of clinically significant alloimmunization occurred among patients with transfusion of non-phenotypically matched blood. Even though there is some discussion on the DNA contamination from residual leukocytes within the transfused donor red cell units, previous studies have showed that DNA from the post transfusion samples does not affect the blood group genotyping results without the risk of detecting microchimerism (Lilian Castilho, et al., 2002; Reid ME, et al., 2000; Rožman, et al., 2000), probably because of the overwhelming excess of patients' DNA. Finding by Reid and associates (Reid ME, et al., 2000) also in agreement with Wenk and Chiafari (Wenk & Chiafari, 1997) who showed that, in 12 massively transfused adult patients, Southern blot analysis of variable number of tandem repeat polymorphism sequences detected patient DNA but not donor DNA.

The results illustrate the importance of performing DNA molecular analysis in the determination of extended blood group genotype amongst multi-transfused patients. The relevance of doing extended blood group genotyping in multiply transfused patients has been demonstrated to be useful by allowing clinicians to determine the actual extended blood group

and assisting in the selection of antigen-negative RBCs for transfusion (Guelsin, et al., 2010). Patients who get benefited by receiving antigen-matched RBCs based on genotype shown better in vivo RBC survival, raises of haemoglobin levels and diminished frequency of transfusion (Lilian Castilho, et al., 2002; Ribeiro, et al., 2009). The haemagglution method, though regarded as the gold standard in blood group identification, the results may be unreliable in certain situation. Many factors need to be considered before interpretation of the results and blood groups assigned. The patient's history of previous exposure to donor blood transfusions, the duration, pregnancy status in females and transplantation should be taken into account especially for vulnerable groups such as infants, the elderly and immunocompromised patients.

Molecular analysis in determining the blood group overcomes the limitation of serology method. DNA testing for red cell blood groups is not influenced by immunoglobulin coating of the red blood cells, the presence of the recently transfused red blood cells or any form of polyagglutination or by the limitations commonly found with the antisera. No mixed- field reaction will occur which lead to the undetermined result. DNA also can be easily extracted from any source such as whole blood, buffy coats, buccal swab or urine sediment and is not influenced by patients' medications or disease condition with the exception of a transplant (J. J. Moulds, 2011). Various studies also showed that DNA from recently transfused blood samples (Reid ME, et al., 2000), urine sediment and buccal swab (Rios, Cash, Strupp, Uehlinger, & Reid, 1999) can be used for blood group molecular genotyping. The technique that applied in the molecular analysis can be fully automated and the results can be analysed, interpreted and documented by computer, which will reduce error in data entry if doing by manual systems. However, caution in performing this test and interpreting the results must be exercised as the PCR-based amplification assays are prone to be contaminated and the genotype determination may not be associated with the antigen expression on the RBCs membrane. Such situations include the detection of genes with a silencing mutation in a location other than that being analysed (point mutation in the GATA box), a gene that is silenced by an alteration of a gene encoding protein with a modifying effect (Rh_{mod} and Rh_{null}) or the failure to detect hybrid genes (particularly in the Rh and MN blood group systems) (Avent & Reid, 2000; Cartron et al., 1998; Cheng-Han Huang & Blumenfeld, 1995; Huang, 1997; Tournamille, Colin, Cartron, & Le Van Kim, 1995).

The development of high-throughput genotyping platforms that utilize microarray and chip technologies offers the opportunity to perform large-scale testing on numerous antigens simultaneously, allowing an accurate selection of donor units to facilitate matching of donor RBCs to the recipient's blood type. Commercial kits available are not only expensive in cost but are developed based on the prevalence of Western population. What is important is the inclusion of alleles that are relevant and prevalent to the Malaysian population. The development of a Malaysian-based genotype assay using molecular techniques suited for small to moderate scale for patients and larger scale for prospective blood donors will be the focus for future research and investigation.

2.4 Figures and Tables

Table 1 Types of cases

Diagnosis	Number of patients
Thalassaemia	
Major	21
Intermedia	32
Hb H Disease	8
Non Thalassaemia	
Congenital Dyserythropoietic Anaemia (CDA)	1
Paroxysmal Nocturnal Haemoglobinuria	1

Table 2 Phenotype and genotype frequencies

	Patients (N = 63)			Patients (N = 63)	
Phenotype (serology)	Frequency	n	Genotype (SNP RT-PCR)	Frequency	n
Rh system					
RhD+	100	63	RHD*+	100	63
RhD-	0	0	RHD*-	0	0
Rh C+c-	42.9	27	RHCE*CC	66.7	42
Rh C+c+	25.4	16	RHCE*Cc	33.3	21
Rh C-c+	3.2	2	RHCE*cc	0	0
Undetermined	28.6	18	Undetermined	0	0
Rh E+e-	1.6	1	RHCE*EE	1.6	1
Rh E+e+	15.9	10	RHCE*Ee	19	12
Rh E-e+	55.6	35	RHCE*ee	79.4	50
Undetermined	27	17	Undetermined	0	0
Kidd system					
Jk(a+b-)	9.5	6	JK*A/JK*A	31.7	20
Jk(a+b+)	33.3	21	JK*A/JK*B	44.4	28
Jk(a-b+)	7.9	5	JK*B/JK*B	23.8	15
Undetermined	49.2	31	Undetermined	0	0
Duffy system					
Fy(a+b-)	12.7	8	FY*A/FY*A	77.8	49
Fy(a+b+)	46.0	29	FY*A/FY*B	19	12
Fy(a-b+)	0	0	FY*B/FY*B	3.2	2
Undetermined	41.3	26	Undetermined	0	0

N, number of individuals; *n*, number of alleles

Table 3 Discrepancies of red cell blood group detected between serology and SNP RT-PCR method

Serological Phenotype	Genotype by SNP RT-PCR			
Rh System	CCee/R1R1	CcEe/R1R2	Ccee/R1r	CcEE/R2Rz
CCee/R1R1	25	1		
CcEe/R1R2	2	7	1	
Ccee/R1r	1		3	
ccEE/R2R2				1
Undetermined	14	4	4	
Kidd System	JKA/JKB	JKA/JKA	JKB/JKB	
Jk (a+b+)	13	5	3	

Jk (a+b-)	1	5	
Jk(a-b+)	1		4
Undetermined	13	10	8
Duffy System	FYA/FYB	FYA/FYA	FYB/FYB
Fy(a+b+)	8	20	1
Fy(a+b-)		8	
Undetermined	4	21	1

The shaded cells indicate the discrepant cases between phenotype by serology and genotype by SNP RT-PCR. All results outside of the shaded cells represent concordances.

3. Conclusion

In conclusion, red blood cell molecular-based genotyping can be helpful in determining the actual extended blood group systems in multiply transfused patient populations and assisting in the identification of suspected antibodies and the selection of antigen-negative RBCs for transfusion.

Acknowledgement

The authors would like to thank the Director General of Health, Ministry of Health Malaysia for granting permission to publish this article.

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