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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. However, this technique has many limitations due to recent exposure to donor red cell, certain drugs or medications or other diseases that may alter the red cell membrane. We aimed to determine the red cell blood group genotype by SNP real time PCR and to compare the results with the conventional serological methods in multiply transfused patients. Sixty-three patients participated in this study whose 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.

1. Introduction

Transfusion management amongst multiply transfused patients are often complicated. The rates of alloimmunization, which is the formation 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 a very complex process due to the presence of donor's blood cells in the patient's blood circulation [1] 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 [2,3]. However, amongst all the blood group systems, the clinically significant groups that have been identified are mainly RH (D, Cc, Ee), KEL (K, k), Kidd (Jk^a, Jk^b), Duffy (Fy^a, Fy^b) [3–5]. 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. While KEL group is indeed clinically significant, due to the homogeneity of this genotype in the Malaysian population, anti-k and anti-K is uncommon as a majority of Malaysians from all ethnic groups are kk [6].

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 [7–12]. In recent years, several molecular methods for RBC typing have been used to

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resolve the problems with haemagglutination test. As the molecular basis of almost all blood group antigens has been determined [13], it is now possible to predict the blood group antigen profile of an individual by testing their DNA with a high degree of accuracy [14,15]. It is even possible to collect DNA from other sources than blood as the genetic code of an individual is contained in all somatic cells [16–19]. Therefore, if there is a possibility of contamination by donor DNA in blood samples, other sources for sampling may be considered.

Determining whether the gene or allele of a particular blood group system is relevant in a particular population is also challenging. There is very little documentation on the extended blood group genotypes in transfusion-dependent patients in Malaysia. However, in most cases where patients have already received multiple transfusions, this would provide more confidence in alloantibody identification procedures and safer transfusions. Identifying the genes to provide a red cell phenotypic profile of the clinically significant blood group antigens would allow for the supply of phenotyped blood as early as possible to reduce the risk of exposure of foreign red cell antigen thus reducing the risk of alloimmunization.

An alternative to serological-based phenotyping is needed to provide the accurate extended blood antigen typing in these patients. Therefore, in the current study, we aimed to compare the phenotype and genotype results from multiply transfused patients. Our objective was to optimize and develop a real-time polymerase chain reaction (RT-PCR) platform using TaqMan® chemistry of single nucleotide polymorphism (SNP) alleles.

2. Materials and methods

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. All of these patients had received 2 or more units of donor RBC previously and had received transfusion within the previous 3–12 weeks. 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. Serological phenotype assignment

The phenotype of the patients' red blood cells was performed by using tube method. Specific antisera were 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.

2.2. SNP RT-PCR assay

3.5 ml of peripheral whole blood was collected. After centrifugation the 200 μl of packed red cells, which also contains the buffy coat layer, was pipetted into a sterile 1.5 ml micro centrifuge tube. The protocol contains a red cell lysis step and wash step to remove the red cells. 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.

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). Both RhE/e and RhC/e were custom-designed while Kidd and Duffy were predesigned assays. The SNP sequences used in this study as shown in Table 1. 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.

Before using the test samples, we examined the SNP RT-PCR method first on 20 donor samples which the red cell profiles have been determined. All experiments were run in duplicate. There was conformity of all antigen profiles tested.

2.3. Sequencing of PCR product

The PCR product was sequenced by a selected company (1st BASE Laboratories Sdn Bhd, Malaysia). Due to the short base pair for each assay tested, the DNA was cloned into competent bacteria. The reference clones were constructed by subcloning of the RHCE, KEL, SLC14A1 and DARC fragments into pJET1.2/vector. Five positive colonies were randomly picked for sequencing. Fig. 1 shows the construct map for the cloning process and Fig. 2 are the detailed sequences of the whole construct.

2.4. Ethical consideration and funding sources

This work 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 work was funded by the Ministry of Higher Education from the Exploratory Research Grant Scheme (grant number ERGS/1/2012/SKK06/USIM/03/1).

3. Results

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 2. 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 may have received during transfusion; filtered red blood cells (FRBC) or in-line filtration units, packed red blood cells (PC) which are plasma-removed red cell units or buffy-coat poor packed cells (BCPPC) which are buffy coat and plasma-removed red cell units. A majority of patients received FRBC products.