Original Article

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Quick Response Code:

Website: www.ajts.org

DOI: 10.4103/ajts.ajts_115_22

A comparison of serological phenotyping and molecular genotyping for Kell, Kidd, and Duffy antigens in multi-transfused thalassemia patients

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Abstract:

BACKGROUND: In multi-transfused thalassemia patients, serological phenotyping fails to test patient's actual blood group antigen profile due to the presence of donor red blood cell (RBC) in the circulation. This limitation of serological tests can be overcome by genotype determination using the polymerase chain reaction (PCR)-based methods. The aim of this study is to compare the serological phenotyping of Kell, Kidd, and Duffy blood group systems with molecular genotyping in the normal blood donors and multi-transfused thalassaemia patients.

MATERIALS AND METHODS: Blood samples from 100 normal blood donors and 50 thalassemia patients were tested using standard serological techniques and PCR-based methods for Kell (K/k), Kidd (Jk^a/Jk^b), and Duffy (Fy^a/Fy^b) blood group systems. The results were compared for concordance.

RESULTS: Genotyping and phenotyping results were 100% concordant for normal blood donors whereas those for thalassemia patients showed 24% discordance. The frequency of alloimmunization in thalassemia patients was 8%. The results of genotyping were used to provide Kell, Kidd, and Duffy matched blood for transfusion therapy to thalassemia patients.

CONCLUSION: The actual antigen profile in multitransfused thalassaemia patients can be reliably determined using genotyping. This would benefit in providing better antigen matched transfusion therapy to such patients hence reducing the rate of alloimmunization.

Keywords:

Alloimmunization, genotype, phenotype, thalassemia

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Submitted: 07-09-2022
Revised: 11-10-2022
Accepted: 06-11-2022
Published: 01-03-2023

Introduction

Red blood cell (RBC) phenotyping by serological methods is often rendered inaccurate in transfusion-dependent patients due to the presence of donor RBCs (mixed cell populations) in peripheral blood for several weeks.[1] In such patients, molecular techniques may be employed in determining the blood antigen type and in antibody identification process.[2] Once the antigen profile of the patient is known, laboratory can ascertain the allo-antibodies which the patient can respond to produce. Similarly, identification of original blood group antigens also facilitates the transfusion of compatible blood units, aiming to minimize the hemolysis of transfused RBCs by these allo-antibodies.

Simple polymerase chain reaction (PCR)-based assays are used to detect a change in a gene encoding a blood group antigen as majority of genetically defined blood group antigens result from a single nucleotide change. The respective antigens for Kell, Kidd, and Duffy blood group systems are almost exclusively a result of single nucleotide polymorphisms (SNP),[3] in strong contrast to the complicated genetic

How to cite this article: Sonker A, Dubey A, Mohan Y. A comparison of serological phenotyping and molecular genotyping for Kell, Kidd, and Duffy antigens in multi-transfused thalassemia patients. Asian J Transfus Sci 2023;17:53-7.

testing for RhD and RhCE or MNSs blood group systems, where additional phenotypic variation is caused by hybrid alleles, resulting from gene conversion events between the two or more highly homologous genes.[4,5]

Molecular testing identifies SNPs and complements the serological typing of blood groups in cases of ambiguous findings. As the genes encoding for majority of blood group systems have been cloned and sequenced, precise area of DNA from patient or donor can be analyzed to predict the presence or absence of a blood group antigen on the surface of an RBC. Studies have shown that white blood cells obtained from the blood samples of recently transfused patients can be used as the source of DNA to determine a blood group polymorphism by PCR-based assays.[6]

In the present study, we have analyzed the correlation of serological (phenotyping) and molecular (genotyping) typing in normal blood donors as well as multitransfused thalassemia patients for Kell, Kidd, and Duffy blood group antigens.

Materials and Methods

This was a prospective observational study carried out after taking the approval from institute's Research and Ethical Committee. The purpose of the study was to ascertain molecular blood group (Kell, Kidd, and Duffy) of multitransfused β thalassemia patients and further to provide Kell, Kidd, Duffy-matched blood to these patients. Fifty random multitransfused (received transfusion of 3 or more blood units in last 3 months) thalassaemia major patients, who had been given blood transfusion with ABO-and RhD-matched blood within 3 weeks were enrolled for this study. Pretransfusion samples of patients before the initiation of blood transfusion therapy were not available. One hundred "O" group regular blood donor samples were also collected and used as controls for the tests. The peripheral blood samples (3 ml in ethylenediaminetetraacetic acid [EDTA] vial and 4 ml in plain vial) of both patients and donors were collected.

Serological analysis

ABO Rh blood grouping of controls and subjects was done by conventional test tube technique using commercially available antisera (Tulip Diagnostics, India). Phenotyping for Kell (K, k), Kidd (Jka, Jkb) and Duffy (Fya, Fyb) antigens was done using commercially available antisera as per the manufacturer's instructions (IMMUCOR, Medizinische Diagnostik GmbH, Germany) by conventional test tube technique. Patients' serum samples were also tested for the presence of allo-antibodies using screening cells (BIORAD, Diamed GmbH, Switzerland). In case of a positive screen, the allo-antibody was further characterized using commercially available reagent red cell panel (BIORAD, Diamed GmbH, Switzerland).

Molecular analysis

Peripheral blood samples collected in EDTA vials were used for DNA extraction. High molecular weight DNA was extracted by using commercially available Qiagen kits (QIAamp DNA Mini Kit; Qiagen Inc., Valencia, CA USA) as per the manufacturer's protocol. The common alleles of Kell, Kidd and Duffy antigens were genotyped using PCR-sequence-specific primer. Briefly, PCR was performed with 50 ng of genomic DNA, 200 µM dNTP, 2 µL 10X PCR buffer, 1.5 mM MgCl2 and 0.5 µL (3U) of Taq DNA Polymerase (AmpliTaq, Perkin Elmer, Branchburg, USA) at a final reaction volume of 20 µl.

Primer and PCR cycling conditions are described in Tables 1 and 2. Known positive and negative controls for different antigens were used for the validation of genotyping assays. The amplification reaction was carried out in thermal cycler (PTC-200 thermal cycler, BioRad, USA) and products were separated by electrophoresis on two per cent agarose gel containing 0.5 µg per mL ethidium bromide. Amplified products were visualized under ultraviolet transilluminator (BIORAD, Diamed GmbH, Switzerland) and the results were documented.

Statistical analysis

To calculate the relative frequency and percentage of different blood group antigens in thalassemia patients, simple statistical calculations were performed using Microsoft Excel 97-2003 worksheet (Microsoft Corporation, U.S.).

Results

In this study, the phenotypes predicted by genotyping were compared with the serological phenotype for Kell, Kidd and Duffy blood group antigen in 100 normal blood donors (control) and 50 patients with β Thalassemia.

Concordance was observed in the results of phenotype and genotype of the blood donors for all the blood group antigens studied [Table 3].

On comparing the genotyping and serological phenotyping results for Kell, Kidd and Duffy blood group antigen in multitransfused thalassemia patients, discrepancies were noticed in 12 (24%) out of 50 patients [Table 4]. For Kell system, there was complete agreement between genotyping and serological phenotyping; 99% of subjects possessed k and 4% possessed K. In Kidd system, out of 15 samples phenotyped as Jk (a + b +) two had Jk^a/Jk^a genotype and three had Jk^b/Jk^b genotype. In Duffy system, out of nine samples phenotyped as Fy (a + b +),

Table 1: Sequence specific primers for Kell, Kidd, and Duffy genotyping

Reaction number	Name of primer	PCR product size	k/K, JK^a/JK^b, and Fy^a/Fy^b PCR-SSP
Common	Kell-672-all-as	-	5'-CGCCAGTGCATCCCTCACC-3'
1	Kell (1)-578-s	140	5'-GACTTCCTTAAACTTTAACCGCAT-3'
2	Kell (2)-578-s	141	5'-GGACTTCCTTAAACTTTAACCGCAC-3'
Common	Kidd-933-all-as	-	5'-GCACAGCCAAGAGCCAGGAGG-3'
3	Kidd (A)-844-Jka-s	131	5'-GTCTTTCAGCCCCATTTGCGG-3'
4	Kidd (B)-844-Jkb-s	131	5'-GTCTTTCAGCCCCATTTGCGA-3'
Common	Duffy(-46)-all-s	-	5'-GCCCTCATTAGTCCTTGGCTCTCAT-3'
5	Duffy (A)-131-as	720	5'-CAGCTGCTTCCAGGTTGCCAC-3'
6	Duffy (B)-131-as	720	5'-CAGCTGCTTCCAGGTTGGTAT-3'
Control	K-HuGroHo-left	434	5'-TGCCTTCCCAACCATTCCCTTA-3'
Control	K-HuGroHo-right	434	5'-CCACTCACGGATTTCTGTTGTGTTTC-3'

PCR=Polymerase chain reaction, SSP=Sequence-specific primer

Table 2: Cycling conditions for polymerase chain reaction-sequence-specific primer based Kell Kidd Duffy genotyping

Steps	PCR conditions	Temperature (°C)	Duration (min: s)	Cycles (repeats)
1	Initial denaturation	94	02:00	×1
2	Denaturation	94	00:10	×10
3	Annealing	65	01:00	
4	Denaturation	94	0:30	×20
5	Annealing	55.9	01:00	
6	Extension	72	00:30	
12	Hold	4	Forever	×1

PCR=Polymerase chain reaction

Table 3: Phenotyping and genotyping results for Kell, Kidd and Duffy systems on samples from 100 normal blood donors

Genotype	Phenotypes			
	K+k−	K−k+	K+k+	
Kell system				
K/k	0	0	4	
K/K	1	0	0	
k/k	0	95	0	
Genotype	**Jk (a+b−)**	**Jk (a+b+)**	**Jk (a−b+)**	**Jk (a−b−)**
Kidd system				
Jk^a/Jk^a	34	0	0	0
Jk^a/Jk^b	0	39	0	0
Jk^b/Jk^b	0	0	26	0
Jk−/Jk−	0	0	0	1
Genotype	**Fy (a+b−)**	**Fy (a+b+)**	**Fy (a−b+)**	
Duffy system				
Fy^a/Fy^a	48	0	0	
Fy^a/Fy^b	0	40	0	
Fy^b/Fy^b	0	0	12	

genotype of six were not in agreement; four sample typed as Fy^a/Fy^a and two as Fy^b/Fy^b. One sample out of 20 Fy (a-b+) phenotypes had Fy^a/Fy^b genotype.

On antibody screening, 4 (8%) samples showed positivity and identification of antibody was done using panel cells [Table 5]. One sample showed the presence of both anti-Jka and anti E. Genotyping and phenotyping discrepancies were concurrent in two samples with alloantibodies.

Discussion

This study aimed to compare genotype with the serological phenotype for Kell, Kidd and Duffy blood group antigens in normal blood donors as well as β Thalassemia patients. There was no discordance in the genotyping and phenotyping of normal blood donors. In β Thalassemia patients who had received blood transfusions, discordance was seen in 24% of samples. The frequency of alloimmunization in the present study was found to be 8% among Thalassemia patients.

Several studies have found that frequently transfused patients have erroneous results on serological phenotyping due to the presence of donors RBC cells in the circulation. Kulkarni *et al*. has reported discrepant results in 77% of thalassemia patients. The authors have compared serological techniques and PCR-based methods for five blood group systems (Rh, Kell, Kidd, Duffy, and MNS). Complete concordance was found between the serological phenotype and genotype for D and k antigens. Maximum discrepancy of 59.1% was observed between genotyping and phenotyping for M, N, S, and s antigens. The study reported 7.5% rate of alloimmunization which is comparable to that in our study.[7]

A study to determine Kidd blood group genotyping for thalassemia patients in Iran found genotype and phenotype discrepancies in 16% cases.[8] The authors have found molecular methods to be a valuable tool to predict blood group phenotypes in multi-transfused patients to select RBC units for a perfect matching improving blood transfusion and preventing alloimmunization.

Castilho *et al*. evaluated the usefulness of blood group genotyping for determining the RBC antigen profile of alloimmunized patients with β-thalassemia and found discrepancy in nine out of ten samples including

Table 4: Genotyping and phenotyping results for Kell, Kidd and Duffy systems on samples from 50 multitransfused thalassemia patients

Genotype	Phenotypes		
	K+k+	K–k+	
Kell system			
K/k	2	0	
k/k	0	48	
Genotype	**Jk (a+b–)**	**Jk (a+b+)**	**Jk (a–b+)**
Kidd system			
Jk^a/Jk^a	20	2*	0
Jk^a/Jk^b	0	10	0
Jk^b/Jk^b	0	3*	15
Genotype	**Fy (a+b–)**	**Fy (a+b+)**	**Fy (a–b+)**
Duffy system			
Fy^a/Fy^a	21	4*	0
Fy^a/Fy^b	0	3	1*
Fy^b/Fy^b	0	2*	19

*Discrepant results

Table 5: Antibodies identified in samples from 50 multitransfused thalassemia patients

Antibody specificity	Frequency	Discrepancy in genotyping and phenotyping
Anti-K	1	No
Anti-Jka*	1	Yes
Anti-Fya	2	Yes (1 sample)
Anti-E*	1	-

*Anti-Jka and anti-E present in same sample

five discrepancies in Rh system. The authors have emphasized on using PCR technology as an alternative to hemagglutination tests to determine antigen profile in cases of alloimmunization.[9]

In a study done by Rujirojindakul and Flegel in Thailand for comparing the serological and molecular typing results in ten patients with thalassaemia, the serological antigen determination was repeated in a reference laboratory after the test was performed at their local blood centre. The serological results of both laboratories differed and even the results obtained in the reference laboratory were often incorrect when cross checked with molecular typing. The authors concluded that molecular blood group phenotype prediction is superior to serological blood group phenotype determination in chronically transfused patients.[10]

Several studies from the past recommend extended antigen matched blood for β Thalassemia patients.[11,12] However, majority of blood transfusion centers rely on serological techniques for RBC phenotyping. There are the high chances of inaccurate results in multi-transfused patients due to the presence of donor RBCs in recipient's circulation. Moreover, since baseline phenotyping before initiation of transfusion therapy is not being done for many patients, it is difficult to provide antigen-matched blood. In such cases, true blood group antigens can be determined by the analysis of DNA. Thus, compatible, or matched units can be selected for transfusion which enhances red cell survival leading to reduction in frequency of blood transfusion. Besides hemoglobinopathies, genotyping of blood group antigens finds application in providing antigen matched packed red blood cells to patients of autoimmune hemolytic anemia and for the resolution of ABO discrepancies.[13] A small margin of error persists even with blood group genotyping as PCR-based techniques are prone to contamination and rarely the genotype may not express the corresponding antigens on RBC membrane. Nevertheless, the potential for incorrect results is lesser in regularly transfused patients than with serological phenotyping.[14]

We found the rate of alloimmunization among thalassemia patients to be 8% which is comparable to other studies from North India where alloimmunization rates vary from 3.8% to 9.48%.[15-17] The specificity of antibodies was determined to be anti E, anti-Jka, anti Fya and anti K. There was simultaneous presence of anti E and anti Jka in one sample. This sample showed genotyping and phenotyping discrepancy as well where the phenotyping was Jk(a + b+) and the genotype was Jk^b/Jk^b. Another sample which had presence of anti-Fya, showed phenotyping as Fy(a + b) and genotyping as Fy^b/Fy^b. Based on genotyping results, we have been able to provide antigen matched blood to our patients. These patients are undergoing regular transfusion therapy and follow up is being done with antibody screening. No new alloimmunization to any red cell antigen has been found and no hemolytic transfusion reaction was reported in these patients. The increment in hemoglobin level posttransfusion was satisfactory. Laboratory parameters associated with red cell lysis such as serum bilirubin, serum lactate dehydrogenase, and reticulocyte counts were also stable in all the patients.

The results of our study reinforce the importance of blood group genotyping in patients requiring repeated blood transfusion. Serological techniques have wider implementation and remain gold standard for routine immunohematology workup, the safety of blood transfusion is greatly enhanced if serological phenotyping is replaced with molecular techniques for reliable antigen determination in multitransfused patients. The authors recommend a further study with larger sample size, clinical correlation with patient's blood transfusion history as well as laboratory parameters and involving greater number of antigens for the determination of factors which lead to discrepancies between genotyping and serological phenotyping.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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