Indian J Med Res 148, December 2018, pp 713-720
DOI: 10.4103/ijmr.IJMR_455_17

Quick Response Code:

Molecular genotyping of clinically important blood group antigens in patients with thalassaemia

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Received March 15, 2017

***Background & objectives*: In multitransfused thalassaemic patients, haemagglutination fails to phenotype the patient's blood group antigens due to the presence of donor-derived erythrocytes. DNA-based methods can overcome the limitations of haemagglutination and can be used to determine the correct antigen profile of these patients. This will facilitate the procurement of antigen-matched blood for transfusion to multitransfused patients. Thus, the aim of this study was to compare the serological phenotyping of common and clinically important antigens of Rh, Duffy, Kell, Kidd and MNS blood group systems with molecular genotyping amongst multitransfused thalassaemic patients.**

***Methods*: Blood samples from 200 patients with thalassaemia and 100 'O' group regular blood donors were tested using standard serological techniques and polymerase chain reaction-based methods for common antigens/alleles (C, c, D, E, e, Fy^a, Fy^b, Jk^a, Jk^b, K, k, M, N, S, s).**

***Results*: Genotyping and phenotyping results were discordant in 77 per cent of thalassaemic patients for five pairs of antithetical antigens of Rh, Duffy, Kell and Kidd blood group systems. In the MNS blood group system, 59.1 per cent of patients showed discrepancy. The rate of alloimmunization among thalassaemics was 7.5 per cent.**

***Interpretation & conclusions*: Molecular genotyping enabled the determination of the actual antigen profile in multitransfused thalassaemia patients. This would help reduce the problem of alloimmunization in such patients and would also aid in the better management of transfusion therapy.**

Key words Blood group genotyping - blood group systems - haemagglutination - molecular genotyping - polymerase chain reaction-sequence-specific primer - thalassaemia

Thalassaemias are genetically inherited blood disorders characterized by defects in the synthesis of globin chain. The goal of transfusion therapy in thalassaemia major patients is to increase the oxygen-carrying capacity by correcting the anaemia, preventing progressive hypersplenism, suppressing erythropoiesis and reducing the increased gastrointestinal absorption of iron.

Patients with thalassaemia need lifelong blood transfusions every 2-4 wk from the early years of life to promote physical growth and general well-being. Patients requiring chronic transfusion support are at higher risk of alloimmunization. The risk depends on recipient's exposure to foreign antigens, their immunogenicity and number and frequency of transfusions and genetic disparity between patient's and donor's antigen profile. The incidence of alloimmunization to red blood cell (RBC) antigens other than ABO and D is particularly high (10-46%) in patients receiving multiple transfusions such as sickle cell disease (SCD) and thalassaemia, and it increases with repeated transfusions[1,2]. The presence of RBC alloantibodies leads to serologic incompatibility, makes the selection of appropriate units for future transfusion difficult, delays the use of a transfusion therapy and presents the risk of haemolytic transfusion reaction. Hence, it is advised that transfusions given to patients who are likely to become transfusion dependent over a long period of time should be matched for antigens other than ABO and RhD.

In multitransfused patients, haemagglutination fails to phenotype the patient's antigens due to donor-derived erythrocytes from previous transfusions. The molecular background of blood group polymorphisms is used for blood group antigen typing[3]. Previous studies have shown that molecular methods prove successful in determining the correct antigen profile of a multitransfused patient[4-6]. 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[4-7]. This approach could significantly reduce the rate of alloimmunization.

The most commonly encountered alloantibodies are produced against the common blood group antigens of Rh, Duffy, Kell and Kidd systems. However, an Indian study reported that after Rh, antibodies against MNS blood group antigens were the most commonly encountered[8]. Hence, in the present study, multitransfused thalassaemic patients were genotyped for clinically important blood group antigens of Rh, Kell, Kidd, Duffy and MNS blood group antigens using DNA-based method and the results were compared with the serological typing results.

Material & Methods

This study was carried out at the department of Transfusion Medicine, ICMR-National Institute of Immunohaematology, Mumbai, India, from January 2013 to December 2015. The study was approved by the Institutional Ethics Committee (project proposal number: EC-GOVT-15/2013). Two hundred consecutive thalassaemia major patients were enrolled for this study. The mean age of the patients was 9.52±6.29 yr (range: 0.5-38 yr) and the average age at the onset of blood transfusion was between six months and 2.5 yr. The patients were transfused every 15-21 days (mean 18 days) with ABO- and RhD-matched blood.

The peripheral blood samples (5 ml) of the patients were collected. Pre-transfusion samples of patients before the first transfusion event were not available. One hundred 'O' group regular blood donor samples were also collected and used as controls (6 RhD-negative and 94 RhD-positive donors). The Rh, Kell, Duffy, Kidd and MNS blood group antigen status was determined in all thalassaemic patients and controls by serological and molecular methods.

Serological analysis: Phenotyping of common Rh (C, c, D, E, e), Duffy (Fy^a, Fy^b), Kell (K, k), Kidd (Jk^a, Jk^b) and MNS (M, N, S, s) antigens was carried out using commercially available antiserum as per manufacturer's instructions (IMMUCOR Inc., USA) by conventional tube technique. Patients' serum samples were also tested for the presence/absence of any atypical antibodies using screening cells. In case of a positive screen, the alloantibody was further characterized using in-house and commercially available reagent red cell panel (BIORAD, DiamedGmbH, Switzerland).

Molecular analysis: Peripheral blood samples with EDTA (ethylenediaminetetraacetic acid) were used for DNA preparation. Genomic DNA was isolated by standard phenol-chloroform/octanol method[9]. The common alleles of Rh, Duffy, Kell, Kidd and MNS antigens were genotyped using polymerase chain reaction-sequence-specific primer (PCR-SSP)[6,10-12]. Briefly, PCR was performed with 100 ng of genomic DNA, 1.5 mM dNTP (BIORON GmbH, Germany), 10X complete buffer (BIORON GmbH, Germany), 25 mM $MgCl_2$ and 0.5 U of Taq DNA Polymerase (BIORON GmbH, Germany) at a final reaction

Table I. List of primers and PCR protocols for genotyping of blood group antigens

Allele specificity	Product size (bp)	Primer name	Sequence 5′ to 3′	PCR protocol	References
Internal control (for RhD)	136	A1	TGTGTTGTAAACCGAGT	95°C for 5 min; 35 cycles of 1 min at 95°C, 1.5 min at 49°C and 2.5 min at 72°C; and finally 1 cycle of 9 min at 72°C	Simsek *et al*, 1995[10]
		A2	ACATGCCATTGCCG		
D	186	A3	TAAGCAAAAGCATCCAA		
		A4	ATGGTGAGATTCTCCT		
C	118	TRH 1	CGCTGCCTGCCCCTCTGC	94°C for 10 min, 30 cycles at 94°C for 30 sec, 58°C for 45 sec and 72°C for 45 sec; 5 min at 72°C	Hojjati *et al*, 2011[6]
		TRH 2	TTGATAGGATGCCACGAGCC		
c	107	TRH 3	CTTGGGCTTCCTCACCTCAAA		
		TRH 4	AAGCCGTCCAGCAGGATTGC		
E	143	TRH 5	TGGCCACGTGTCAACTCTC		
		TRH 7	CATGCTGATCTTCCTTTGGG		
e	143	TRH 6	TGGCCACGTGTCAACTCTG		
		TRH 7	CATGCTGATCTTCCTTTGGG		
FY common		Duffy(-46) -all-as	GCCCTCATTAGTCCTTGGCTCTCAT	120 sec at 94°C; 10 incubation cycles for 10 sec at 94°C and 60 sec at 65°C and 20 incubation cycles for 30 sec at 94°C, 60 sec at 61°C and 30 sec at 72°C	Rozman *et al*, 2000[11]
Fy^a	720	Duffy (A)-131-as	CAGCTGCTTCCAGGTTGCCAC		
Fy^b	720	Duffy (B)-131-as	CAGCTGCTTCCAGGTTGGTAT		
JK common		Kidd-933-all-as	GCACAGCCAAGAGCCAGGAGG		
Jk^a	131	Kidd (A)-844-Jka-s	GTCTTTCAGCCCCATTTGCGG		
Jk^b	131	Kidd (B)-844-Jkb-s	GTCTTTCAGCCCCATTTGCGA		
KEL common		Kell-672-all-as	CGCCAGTGCATCCCTCACC		
K1	140	Kell (1)-578-s	GACTTCCTTAAACTTTAACCGCAT		
K2	141	Kell (2)-578-s	GGACTTCCTTAAACTTTAACCGCAC		
Internal control (same for C, c, E and e also)	434	Oligo, K-HuGroHo-left	TGCCTTCCCAACCATTCCCTTA		
		Oligo, K-HuGroHo-right	CCACTCACGGATTTCTGTTGTGTTTC		
M1	432	Mf2	AATTGTGAGCATATCAGCATC	2 min at 94°C; 10 cycles of 10 sec at 94°C and 1 min at 65°C and 20 cycles of 10 sec at 94°C, 50 sec at 61°C and 30 sec at 72°C	Heymann and Salama, 2010[12]
		Mr2	GGGTCTGAGCTGAACTCAG		
M2	262	Mf2	AATTGTGAGCATATCAGCATC		
		NMr1	GCAAGAATTCCTCCATAGTAG		
M3	250	Mf3	CAGCATCAAGTACCACTGGT		
		NMr1	GCAAGAATTCCTCCATAGTAG		

Contd...

Allele specificity	Product size (bp)	Primer name	Sequence 5′ to 3′	PCR protocol	References
M4	109	NMf1	CAAAGCACAGAAATGATGCAC		
		Mr2	GGGTCTGAGCTGAACTCAG		
N1	262	Nf2	AATTGTGAGCATATCAGCATT		
		NMr1	GCAAGAATTCCTCCATAGTAG		
N2	250	Nf3	CAGCATTAAGTACCACTGAG		
		NMr1	GCAAGAATTCCTCCATAGTAG		
S1	128	gSas	ACGATGGACAAGTTGTCCCA		
		SSf1	TGATTAAGAAAAGGAAACCCG		
S2	128	Ksas	CGATGGACAAGTTGTCCCG		
		SSf1	TGATTAAGAAAAGGAAACCCG		
Internal control	629	Forward	GCCTTCCCAACCATTCCCTT		
		Reverse	TAGACGTTGCTGTCAGAGGC		

volume of 25 µl. Primer and PCR cycling conditions are described in Table I. 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 (Veriti® 96-well Thermal Cycler, Applied Biosystems, USA) and products were separated electrophoretically on two per cent agarose gel containing ethidium bromide. Amplified products were visualized under ultraviolet transilluminator, Gel Doc system (BIORAD, DiamedGmbH, Switzerland).

Statistical analysis: To calculate the relative frequency (Mean and percentage) of different blood group antigens in thalassaemic individuals, appropriate simple statistical tests were performed.

Results

In the present study, alloimmunization was observed in 15 of 200 multitransfused thalassaemic patients (7.5%). In seven patients, antibodies of single specificity were detected [anti-C (1), anti-c (3), anti-E (2) and anti-K (1)] and in the remaining patients, antibodies reacted with all the panel cells. PCR-SSP method was standardized and the genotype was determined for common antigens of Rh, Duffy, Kell, Kidd and MNS antigens (Fig. 1). Among donors, the RH, KEL, FY, JK and MNS genotypes were concordant with all the corresponding serological phenotypes. The genotype frequencies observed in the two groups studied (thalassaemic patients and blood donors) showed no significant differences.

The genotyping in thalassaemic patients was concordant with the serological red cell phenotype in only 46 (23%) patients for five antithetical pairs of antigens belonging to four blood group systems (Rh, Duffy, Kell and Kidd). The remaining patients (77%) showed phenotype/genotype discrepancies. Of these 154 patients giving discrepant results, there were 63 patients with one (40.9%), 54 patients with two (35%), 26 with three (16.9%), 10 with four (6.5%) and one patient with five (0.7%) antigen discrepancies. The antigens of Rh system (c, E) showed the maximum discrepancy between genotyping and serologic phenotyping (Fig. 2). The post-transfusion genotyping results were not influenced by the number of transfusions given or sampling period after transfusion. In Kidd system, phenotype/genotype results did not show concordance in 77 patients. There was an agreement between phenotype and

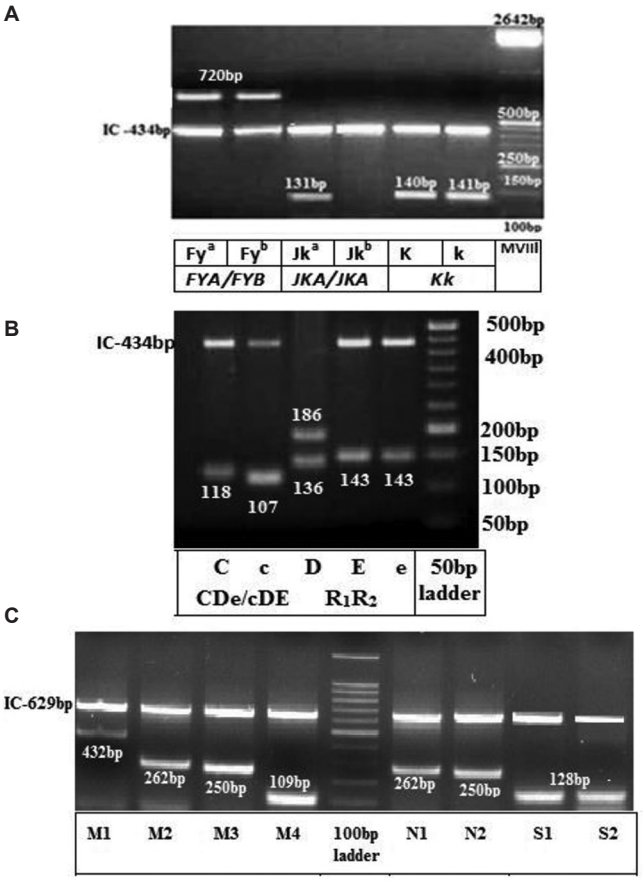

Fig. 1. Standardization of RBC antigen genotyping by PCR-SSP (**A**) FY1, FY2, JK1, JK2, K1 and K2 alleles; (**B**) C, c, D, E, and e antigens; (**C**) M, N, S and s antigens: M1, M2, M3 and M4 represent amplification of different regions of GYPA responsible for M antigen specificity (as followed by Heymann and Salama, 2010)[12]. Presence of all the specific fragments accounts for the presence of M antigen. Similarly, N1 and N2 represent amplification of two regions of GYPA responsible for N antigen specificity. S/s polymorphism is due to a SNP, hence a single PCR each for S and s has been used for genotyping. PCR, polymerase chain reaction; SSP, sequence-specific primer; SNP, single-nucleotide polymorphism; RBC, red blood cell.

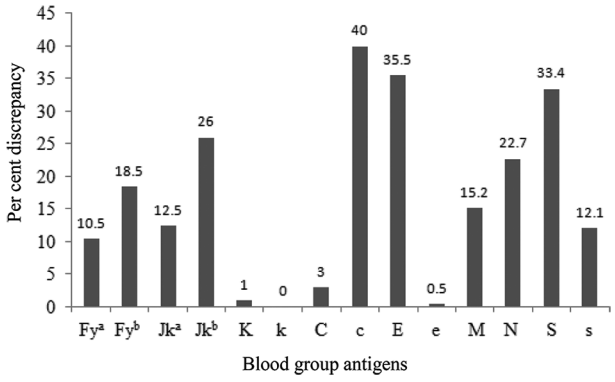

Fig. 2. Percentage discrepancy of clinically important blood group antigens by serotyping and genotyping among thalassaemia patients.

genotype results for Duffy blood group antigens in 142 of the 200 patients. Two patients showing Kk as the phenotype were genotyped and found to be *K2/K2*. Similarly, four patients phenotyped as RHCc were genotyped as RHCE*cc. There was a complete concordance between the serological phenotype and genotype for D and k antigens. The antigens of the Rh system (c, E) showed maximum discrepancy between genotyping and serologic phenotyping in thalassaemic patients tested. A total discrepancy of 59.1 per cent was observed between genotyping and phenotyping for M, N, S and s antigens. In MNS system, antigens N and S showed maximum discrepancy of 22.7 and 33.4 per cent, respectively (Fig. 2).

In 80 per cent of alloimmunized cases (n=12), who showed discrepant results, there were eight patients with one, one patient with two and two patients with three and one patient with four antigen discrepancies. Overall discrepancy was found among eight antigens (Fy^a, Fy^b, Jk^a, Jk^b, K, C, c, E). In these patients, 20 per cent discrepancy was found for Jk^b, c and E antigens each. Fy^a and Fy^b accounted for 10 and 15 per cent discrepancy respectively, while Jka and K accounted for 5 per cent discrepancy each.

Discussion

Development of red cell alloantibodies is a common complication in patients undergoing chronic blood transfusion therapy. In blood banks, only ABO and RhD grouping is performed as a part of pre-transfusion testing. Hence, the incidence of alloimmunization to other RBC antigens in multitransfused patients is particularly high (5-33%) and can cause problems in transfusion management[1,13-15]. The most important unexpected RBC alloantibodies are directed towards the Rh (D, C, E, c and e) and Kell (K) antigens, followed by antigens of the Duffy, Kidd and MNS blood group systems. Studies have shown that transfusion with phenotype-matched units has greatly reduced the rate of alloimmunization in thalassaemics and SCD patients along with improved RBC survival and diminished frequency of transfusions[1,16-18].

Accurate antigen typing by serology is difficult in case of multitransfused patients due to the presence of donor RBCs in patient's circulation. In the present study, simple PCR-based assays were used for detecting alleles/antigens of the Rh, Duffy, Kell, Kidd

Table II. Incidence of alloimmunization in thalassaemic patients from different parts of India

Region	Author, year and reference number	Incidence of alloimmunization (%)	Specificity of antibody detected
Navi Mumbai	Gupta and Mehra, 2016[23]	5.26	Anti-D, Anti-C, Anti-E, MNS, Kell
Chandigarh	Jain *et al*, 2016[24]	3.3	Anti-D, Anti-c, Anti-Jk^b+E, Kell
Surat	Patel *et al*, 2016[25]	8.0	Rh, Kell, Kidd, Duffy, MNS, Lewis, P
New Delhi and Karnataka	Agrawal *et al*, 2016[26]	2.91	Anti-C, Anti-K
West Bengal	Datta *et al*, 2015[27]	5.6	Anti-D, Anti-C, Anti-c, Anti-E, Anti-s, Anti-Jk^a, Anti-Jk^b Anti-C+D, Anti-E+Jk^b, Anti-E+Fy^b
Jammu	Dogra *et al*, 2015[28]	8.5	Anti-D, Anti-E, Anti-K
Haryana	Dhawan *et al*, 2014[29]	5.64	Anti-D, Anti-C, Anti-C^w, Anti-E, Anti-K, Anti-Jk^b, Anti-Xg
Pune	Philip *et al*, 2014[30]	5.5	Anti-D, Anti-E, Anti-c, Anti-M, Anti-Le^a, Anti-S
Bengaluru	Shenoy *et al*, 2013[31]	9.46	Anti-C, Anti-E, Kell, Duffy
Delhi	Gupta *et al*, 2011[2]	9.48	Anti-E, Anti-C^w, Anti-K, Anti-Kp^a
Mumbai	Pradhan *et al*, 2001[14]	8.0	Rh
Present study		7.5	Anti-C, anti-c, anti-E, anti-K

and MNS blood group systems. These molecular methods allowed rapid and accurate identification of blood group antigens. Among the regular blood donors, there was complete concordance in phenotype deduced by haemagglutination and genotype deduced by molecular methods. In thalassaemics, discrepancy between phenotype and genotype for Rh, Kell, Duffy and Kidd system antigens was found to be 77 per cent, of which 40.9 per cent showed one antigen discrepancy, while the remaining showed more. One patient had five antigen discrepancies. For MNS blood group antigens, discrepancy was noted in 59.1 per cent. Our findings were comparable to varied range of discrepancy (15-90%) reported worldwide between genotype and phenotyping results among multitransfused patients[18-22]. In 40 multiply transfused SCD patients, 15 per cent discrepancy was found for antigens of the Rh (D, C/c, E/e), Kell, Kidd and Duffy systems when tested by haemagglutination and PCR-restriction fragment length polymorphism[18]. In Lithuanian patients, 33 per cent disagreements were reported in Fy^a and Fy^b typing, while thalassaemic patients from Thailand showed 90 per cent discrepancy for five antithetical antigen pairs in four blood group systems[19,20]. In a study from the USA, of the 16 patients studied, four (one SCD patient and three thalassaemics) demonstrated multiple antigen (M, c, E, K, Jk^a and Jk^b) discrepancies[21]. Among 200 thalassaemic patients studied, 71 per cent discrepancy between phenotype and genotype was reported in Duffy system, 38.5 per cent in Kidd system and 53.5 per cent cases in the Rh blood group system which was comparable for Duffy and Rh but lower for Kidd antigens in a Malaysian study[22]. Our findings also showed higher total discrepancy rate (77%), although the results were not directly compared with others due to different ethnic background, variable sample size, different age group and difference in RBC units transfused.

In our study, the rate of alloimmunization was 7.5 per cent, which was comparable to other Indian studies (Table II)[2,14,23-31]. Single-specific alloantibodies were detected among seven patients. In one of the patients, anti-c antibody was identified. By serology, the Rh phenotype of the patient was R_1r, but by molecular methods, it was confirmed as R_1R_1. Serological results indicated the presence of 'c' antigen against which anti-c antibody was produced by the patient. However, molecular genotyping confirmed the absence of 'c' antigen in the patient, thus revealing the actual antigen profile of the patient and also helped in confirming the allo-specificity of anti-c antibody in patient's serum.

In conclusion, molecular genotyping was found to be an accurate reliable method for minor antigen typing and should be used for providing antigen-negative or antigen-matched blood units to multitransfused thalassaemic patients. In the present study,

genotype:phenotype discrepancies were observed in 77 per cent of multitransfused thalassaemics and genotyping enabled the determination of the actual antigen profile. Further, simple PCR-SSP assays can be easily performed in any hospital having molecular biology laboratory.

***Financial support & sponsorship*:** The authors acknowledge the Indian Council of Medical Research, New Delhi, India for financial support.

***Conflicts of Interest*:** None.

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