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

Swati Kulkarni, Bhavika Choudhary, Harita Gogri, Shashikant Patil, Mamta Manglani, Ratna Sharma & Manisha Madkaikar

1Department of Transfusion Medicine, ICMR-National Institute of Immunohaematology, KEM Hospital Campus, 2Pediatric Hematology-Oncology & BMT Centre, Lokmanya Tilak Municipal General Hospital, Mumbai & 3Arpan Blood Bank, Nashik, India

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 and Kidd 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, Fyα, Fyβ, Jkα, Jkβ, 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. 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. Previous studies have shown that molecular methods prove successful in determining the correct antigen profile of a multitransfused patient. 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. 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. Hence, in the present study, multitransfused thalassaemic patients were genotyped for clinically important blood group antigens of Rh, Kell, Kidd 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-GOV-T-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\textsuperscript{a}, Fy\textsuperscript{b}), Kell (K, k), Kidd (Jk\textsuperscript{a}, Jk\textsuperscript{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. The common alleles of Rh, Duffy, Kell, Kidd and MNS antigens were genotyped using polymerase chain reaction-sequence-specific primer (PCR-SSP). 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.
| Allele specificity | Product size (bp) | Primer name | Sequence 5′ to 3′ | PCR protocol | References |
|--------------------|------------------|-------------|-------------------|--------------|------------|
| Internal control (for RhD) | 136 | A1 | TGTGTGTGATAACCCAGT | 95°C for 5 min; 35 cycles | Simsek et al., 1995 |
| | | A2 | ACATGCCATTGCGC | | |
| D | 186 | A3 | TAAGCAAAAGCATCACA | 94°C for 10 min, 30 cycles | Hojjati et al., 2011 |
| | | A4 | ATGGTGAGATTCTCCTTCT | | |
| C | 118 | TRH 1 | CGCTGCCTGCCCTCTGC | 94°C for 10 min, 30 cycles | Rozman et al., 2000 |
| | | TRH 2 | TTGATAGGATGCAAGCAGGCC | | |
| | | TRH 3 | CTTGGGCTTCTCACCTCAA | | |
| | | TRH 4 | AAGCCGTCCAGCAGGATTGC | | |
| E | 143 | TRH 5 | TGGCCACGTTCAACTCTC | | |
| | | TRH 6 | CATGCTGATCTCTTCTTGGG | | |
| | | TRH 7 | CATGCTGATCTCTTCTTGGG | | |
| FY common | 720 | Duffy(-46)-all-as | GCCCTCATTAGTCTTGGCTCTCAT | 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 |
| | | Duffy (A)-131-as | CAGCTGCTTTCCAGGTTGCAC | | |
| | | Duffy (B)-131-as | CAGCTGCTTTCCAGGTTGCAC | | |
| JK common | Kidd-933-all-as | GCAAGCAGCAAGGCGAGG | | |
| | | Kidd (A)-844-Jka-s | GTCTTTCAGCCCATTTGCG | | |
| | | Kidd (B)-844-Jkb-s | GTCTTTCAGCCCATTTGCG | | |
| KEL common | Kel-672-all-as | GCCGAGTCATCCCTCAACC | | |
| K1 | 140 | Kel (1)-578-s | GACTTTCCTTAAACTTTAACCAGCAT | | |
| K2 | 141 | Kel (2)-578-s | GACTTTCCTTAAACTTTAACCAGCAT | | |
| Internal control (same for C, c, E and e also) | 432 | Oligo, K-HuGroHo-left | TGCTTTCCTCA ACCATCCCTTA | 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 |
| | | Oligo, K-HuGroHo-right | CCATCACAGGATTTGTGTGTTTC | | |
| M1 | 432 | Mr2 | AATTGTGAGCATATCAGCATC | | |
| | | Mr2 | GGTTCTAGCTGAACTCAG | | |
| M2 | 262 | Mr2 | AATTGTGAGCATATCAGCATC | | |
| | | NMr1 | GCAAGAATTCTCTCCATAGTAG | | |
| M3 | 250 | Mr3 | CAGCATCAGTTCCAATCCTGT | | |
| | | NMr1 | GCAAGAATTCTCTCCATAGTAG | | |

*Contd...*
| Allele specificity | Product size (bp) | Primer name | Sequence 5′ to 3′ | PCR protocol | References |
|--------------------|------------------|-------------|-------------------|--------------|------------|
| M4                 | 109              | NMf1        | CAAGACAGAAGATGGCAC | PCR          |            |
|                    |                  | Mr2         | GGGTCTGAGCTGAACTCAG |              |            |
| N1                 | 262              | Nf2         | AATTGTGAGCATATCAGTAT | PCR          |            |
| N2                 | 250              | NMf2        | CAAGAACCTGCAATCTAG |              |            |
|                    |                  | NMr1        | GCAAGAATTCCTCCATAGTAG |              |            |
| S1                 | 128              | gSas        | ACGATGGACAAGTTGTCCCA | PCR          |            |
| S2                 | 128              | SSf1        | CGATGGACAAGTTGTCCCG |              |            |
| Internal control   | 629              | Forward     | GCTTCTGCCAACATTCTTTT | PCR          |            |
|                    |                  | Reverse     | TAGACGTTGCTGTCAGGCCG |              |            |

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
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 RHCE were genotyped as RHCEcc. 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 (Fya, Fyb, Jka, Jkb, K, C, c, E). In these patients, 20 per cent discrepancy was found for Jkb, c and E antigens each. Fya and Fyb 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 management1,13-15. 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 transfusions1,16-18.

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
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.

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. In Lithuanian patients, 33 per cent disagreements were reported in Fy\textsuperscript{a} and Fy\textsuperscript{b} typing, while thalassaemic patients from Thailand showed 90 per cent discrepancy for five antithetical antigen pairs in four blood group systems. 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\textsuperscript{a} and Jk\textsuperscript{b}) discrepancies. 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. 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. 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\textsuperscript{1r}, but by molecular methods, it was confirmed as R\textsuperscript{1r} R\textsuperscript{1r}. 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,

| Region                  | Author, year and reference number | Incidence of alloimmunization (%) | Specificity of antibody detected |
|-------------------------|-----------------------------------|----------------------------------|----------------------------------|
| Navi Mumbai             | Gupta and Mehra, 2016\textsuperscript{23} | 5.26                             | Anti-D, Anti-C, Anti-E, MNS, Kell |
| Chandigarh              | Jain et al, 2016\textsuperscript{16} | 3.3                              | Anti-D, Anti-c, Anti-Jk\textsuperscript{b}+E, Kell |
| Surat                   | Patel et al, 2016\textsuperscript{25} | 8.0                              | Rh, Kell, Kidd, Duffy, MNS, Lewis, P |
| New Delhi and Karnataka | Agrawal et al, 2016\textsuperscript{16} | 2.91                             | Anti-C, Anti-K                   |
| West Bengal             | Datta et al, 2015\textsuperscript{27} | 5.6                              | Anti-D, Anti-C, Anti-c, Anti-E, Anti-s, Anti-Jk\textsuperscript{a}, Anti-Jk\textsuperscript{b} Anti-C\textsuperscript{+}D, Anti-E+Jk\textsuperscript{b}, Anti-E+Fy\textsuperscript{b} |
| Jammu                   | Dogra et al, 2015\textsuperscript{28} | 8.5                              | Anti-D, Anti-E, Anti-K           |
| Haryana                 | Dhawan et al, 2014\textsuperscript{29} | 5.64                             | Anti-D, Anti-C, Anti-C\textsuperscript{a}, Anti-E, Anti-K, Anti-Jk\textsuperscript{b}, Anti-Xg |
| Pune                    | Philip et al, 2014\textsuperscript{30} | 5.5                              | Anti-D, Anti-E, Anti-c, Anti-M, Anti-Le\textsuperscript{a}, Anti-S |
| Bengaluru               | Shenoy et al, 2013\textsuperscript{31} | 9.46                             | Anti-C, Anti-E, Kidd, Duffy      |
| Delhi                   | Gupta et al, 2011\textsuperscript{12} | 9.48                             | Anti-E, Anti-C\textsuperscript{a}, Anti-K, Anti-Kp\textsuperscript{a} |
| Mumbai                  | Pradhan et al, 2001\textsuperscript{14} | 8.0                              | Rh                               |
| Present study           |                                    | 7.5                              | Anti-C, anti-c, anti-E, anti-K   |
genotype:phenotype discrepancies were observed in 77 per cent of multitransfused thalassaemias 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.

References
1. Singer ST, Wu V, Mignacca R, Kuypers FA, Morel P, Vichinsky EP. Alloimmunization and erythrocyte autoimmunization in transfusion-dependent thalassemia patients of predominantly Asian descent. Blood 2000; 96 : 3369-73.
2. Gupta R, Singh DK, Singh B, Rusia U. Alloimmunization to red cells in thalassemias: Emerging problem and future strategies. Transfus Apher Sci 2011; 45 : 167-70.
3. Reid ME, Rios M, Powell VI, Charles-Pierre D, Malavade V. DNA from blood samples can be used to genotype patients who have recently received a transfusion. Transfusion 2000; 40 : 48-53.
4. Avent ND. Large-scale blood group genotyping: Clinical implications. Br J Haematol 2009; 144 : 3-13.
5. Guelsin GA, Sell AM, Castilho L, Masaki VL, Melo FC, Hashimoto MN, et al. Benefits of blood group genotyping in multi-transfused patients from the South of Brazil. J Clin Lab Anal 2010; 24 : 311-6.
6. Hojjati MT, Einollahi N, Nabatchian F, Pourfathollah AA, Mahdavi MR. Allele-specific oligonucleotide polymerase chain reaction for the determination of Rh C/c and Rh E/e antigens in thalassaemic patients. Blood Transfus 2011; 9 : 301-5.
7. Westhoff CM. The potential of blood group genotyping for transfusion medicine practice. Immunohematology 2008; 24 : 190-5.
8. Gogri H, Kulkarni S, Vasantha K, Jadhav S, Ghosh K, Gorakshakar A. Partial matching of blood group antigens to reduce alloimmunization in Western India. Transfus Apher Sci 2016; 54 : 390-5.
9. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
10. Simsek S, Faas BH, Bleeker PM, Overbeeke MA, Cuipers HT, van der Schoot CE, et al. Rapid Rh D genotyping by polymerase chain reaction-based amplification of DNA. Blood 1995; 85 : 2975-80.
11. Rozman P, Dovc T, Gassner C. Differentiation of autologous ABO, RHD, RHCE, KEL, JK, and FY blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions. Transfusion 2000; 40 : 936-42.
12. Heymann GA, Salama A. Sequence-specific primers for MNS blood group genotyping. Blood Transfus 2010; 8 : 159-62.
13. Ho HK, Ha SY, Lam CK, Chan GC, Lee TL, Chiang AK, et al. Alloimmunization in Hong Kong Southern Chinese transfusion-dependent thalassemia patients. Blood 2001; 97 : 3999-4000.
14. Pradhan V, Badakere S, Vasantha K, Korgeonkar S, Panjwani S, Jawo N. Antibodies to red cells in beta thalassemia major patients receiving multiple transfusions: A short report. Indian J Hematol Blood Transfus 2001; 19 : 100-1.
15. Jain R, Choudhury N, Chudgar U, Harimooorthy V, Desai P, Perkins J, et al. Detection and identification of red cell alloantibodies in multiply transfused thalassemia major patients: A prospective study. Indian J Hematol Blood Transfus 2014; 30 : 291-6.
16. Vichinsky EP, Luban NL, Wright E, Olivieri N, Driscoll C, Pegelow CH, et al. Prospective RBC phenotype matching in a stroke-prevention trial in sickle cell anemia: A multicenter transfusion trial. Transfusion 2001; 41 : 1086-92.
17. Pujani M, Pahuja S, Dhingra B, Chandra J, Jain M. Alloimmunisation in thalassaemia: A comparison between recipients of usual matched and partial better matched blood. An evaluation at a tertiary care centre in India. Blood Transfus 2014; 12 (Suppl 1) : s100-4.
18. Castilho L, Rios M, Bianco C, Pellerino J Jr., Alberto FL, Saad ST, et al. DNA-based typing of blood groups for the management of multiply-transfused sickle cell disease patients. Transfusion 2002; 42 : 232-8.
19. Remeikiene D, Ugenksiene R, Inciura A, Savukatiyte A, Raulinaityte D, Skrodeniene E, et al. Duffy and Kidd genotyping facilitates pretransfusion testing in patients undergoing long-term transfusion therapy. Turk J Haematol 2014; 31 : 367-73.
20. Ruijroijindakul P, Flegel WA. Applying molecular immunohaematology to regularly transfused thalassaemic patients in Thailand. Blood Transfus 2014; 12 : 28-35.
21. Ye Z, Zhang D, Boral L, Liz C, May J. Comparison of blood group molecular genotyping to traditional serological phenotyping in patients with chronic or recent blood transfusion. J Biosci Med 2016; 4 : 1.
22. Osman NH, Sathar J, Leong CF, Zulkifli NF, Raja Sabudin RZA, Othman A, et al. Importance of extended blood group genotyping in multiply transfused patients. Transfus Apher Sci 2017; 56 : 410-6.
23. Gupta S, Mehra R. Red cell allo-immunization in thalassemia patients. J Evol Med Dent Sci 2016; 5 : 2681-4.
24. Jain A, Agnihotri A, Marwaha N, Sharma RR. Direct antiglobulin test positivity in multi-transfused thalassemias. Asian J Transfus Sci 2016; 10 : 161-3.
25. Patel AS, Gamit S, Gohil M. Role of RBC’s alloimmunization in multiple transfused thalassaemia patients. Int J Res Med Sci 2016; 4 : 822-8.
26. Agrawal A, Mathur A, Dontula S, Jagannathan L. Red blood cell alloimmunization in multi-transfused patients: A bicentric study in India. Global J Transfus Med 2016; 1 : 12.
27. Datta SS, Mukherjee S, Talukder B, Bhattacharya P, Mukherjee K. Frequency of red cell alloimmunization and autoimmunization in thalassemia patients: A report from Eastern India. *Adv Hematol* 2015; 2015: 610931.

28. Dogra A, Sidhu M, Kapoor R, Kumar D. Study of red blood cell alloimmunization in multitransfused thalassemic children of Jammu region. *Asian J Transfus Sci* 2015; 9: 78-81.

29. Dhawan HK, Kumawat V, Marwaha N, Sharma RR, Sachdev S, Bansal D, *et al*. Alloimmunization and autoimmunization in transfusion dependent thalassemia major patients: Study on 319 patients. *Asian J Transfus Sci* 2014; 8: 84-8.

30. Philip J, Biswas AK, Hiregoudar S, Kushwaha N. Red blood cell alloimmunization in multitransfused patients in a tertiary care center in Western India. *Lab Med* 2014; 45: 324-30.

31. Shenoy B, Voona MM, Shivaram C, Nijaguna S. Red cell alloimmunization in multitransfused patients with beta thalassemia major - A study from South India. *Int J Med Pharm Sci* 2013; 3: 31-40.

*For correspondence:* Dr Swati Kulkarni, Department of Transfusion Medicine, ICMR-National Institute of Immunohaematology, 13th Floor, New Multi-Storeyed Building, KEM Hospital Campus, Parel, Mumbai 400 012, Maharashtra, India

e-mail: swatiskulkarni@gmail.com