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Molecular typing of Kell, Kidd, and Duffy antigens in direct antiglobulin test-positive autoimmune hemolytic anemia patients

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Abstract:

BACKGROUND: In autoimmune hemolytic anemia (AIHA) patients, conventional pretransfusion testing is difficult to interpret due to the presence of autoantibodies which may show panreactivity. Molecular phenotyping of red cell antigens could potentially be used to precisely match blood units, thereby reducing the need to perform intensive serologic laboratory testing, hence time delay in providing transfusion to such patients. The aim of this study is to perform the molecular typing for Kell, Kidd, and Duffy blood group antigens in direct antiglobulin test (DAT)-positive red blood cells of AIHA patients and provide corresponding antigen-matched blood for transfusion therapy.

MATERIALS AND METHODS: Blood samples from 50 normal blood donors and 30 DAT-positive AIHA patients were tested using standard serological techniques and polymerase chain reaction-based methods for Kell (K/k), Kidd (Jk^a/Jk^b), and Duffy (Fy^a/Fy^b) blood group systems. Five patients requiring blood transfusion were given donor blood units identical for Kell, Kidd, and Duffy antigens and followed up.

RESULTS: Genotyping and phenotyping results were 100% concordant for normal blood donors. Serological phenotyping of minor red cell antigens showed varied degree of agglutination for AIHA patients. The molecular typing was able to detect the antigen frequency accurately in all samples. The results of genotyping were used to provide Kell-, Kidd-, and Duffy-matched blood for transfusion therapy to AIHA patients with no adverse reaction.

CONCLUSION: Molecular blood group typing has proved immensely useful in the determination of actual antigen profile and hence in providing appropriate transfusion support in patients with AIHA reduced risk of transfusion reactions and alloimmunization.

Keywords:

Autoimmune hemolytic anemia, autoantibody, blood transfusion, genotype, phenotype

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Introduction

Autoimmune hemolytic anemia (AIHA) is a variety of disorders, in which autoantibodies are formed against antigens on the red blood cell (RBC), leading to shortened survival of patients' own as well as transfused RBCs. There are three broad categories of autoantibodies which show varied serologic properties, leading to distinct clinical manifestations. Warm autoantibodies are IgG type which attach to erythrocytes at 37°C, cold autoantibodies are IgM type which clump RBCs at cold temperatures, and Donath–Landsteiner antibodies are IgG type which bind to RBC membranes in the cold and result in hemolysis at 37°C.[1]

It is particularly challenging to provide compatible blood units to patients who have red cell autoantibodies in their serum

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because the presence of autoantibody may mask the detection of an underlying alloantibody. Therefore, knowing of the patient's predicted phenotype through molecular typing of the blood group antigens is useful for determining the alloantibodies which he or she can produce. This may also be helpful for selecting the RBCs for heterologous adsorption of the autoantibody. The autoantibodies lead to strongly reactive direct antiglobulin test (DAT), rendering the serological typing of red cells difficult. Moreover, it is tedious to determine serological phenotypes in patients who have been recently transfused.[2]

The phenotype prediction through the application of molecular techniques is particularly valuable for the management of patients who have reactive warm autoantibodies. Molecular phenotyping of red cell antigen could potentially be used to precisely match blood units, thereby reducing the need to perform intensive serologic laboratory testing, thus reducing the time delay in providing transfusion to such patients.[3]

The present study has been carried out with the objectives of performing the molecular typing for Kell, Kidd, and Duffy blood group antigens in DAT-positive RBCs of AIHA patients and provides corresponding antigen-matched blood based for transfusion therapy to these patients.

Materials and Methods

This was a prospective observational study carried out after taking the approval from the institute's research and ethical committee. The purpose of the study was to determine molecular blood group (Kell, Kidd, and Duffy) of DAT-positive AIHA patients and further to provide Kell-, Kidd-, and Duffy-matched blood to these patients. As this is a pilot exploratory research study, nonprobability sampling was done. As per the guidelines for sample size calculation, nonstatistical methods should be applied for sample size calculation and 20–150 participants may be chosen for such studies.[4] For our study, 30 random DAT-positive patients, whose samples were sent for serological evaluation and who had been given blood transfusion with ABO- and Rh D-matched blood within 3 weeks, were enrolled for this study. Pretransfusion samples of patients before the initiation of blood transfusion therapy were not available. Fifty "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 (Jk^a, Jk^b), and Duffy (Fy^a, Fy^b) antigens was done using commercially available antisera as per the manufacturer's instructions (IMMUCOR, Medizinische Diagnostik GmbH, Germany) by the conventional test tube technique. Patients' serum samples were also tested for DAT using polyspecific IgG + C3d LISS-Coombs cards (BIORAD, Diamed GmbH, Switzerland) and for the presence of alloantibodies by indirect antiglobulin test (IAT) using screening cells (BIORAD, Diamed GmbH, Switzerland). In case of positive DAT, elution was done using Cold-acid elution procedure.[5] The eluate was further tested with reagent red cell panel (BIORAD, Diamed GmbH, Switzerland) to determine the reactivity. Supernatant from the final wash of red cells eluted was also tested in parallel along with eluate. Its nonreactivity ensured that antibody detected in eluate was only red cell-bound antibody and not free antibody from the plasma. In case of a positive screen, the alloantibody was further characterized using commercially available reagent red cell panel.

Molecular analysis

Peripheral blood samples collected in EDTA vials were used for DNA extraction. High-molecular weight DNA was extracted 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 the polymerase chain reaction-sequence-specific primer (PCR-SSP). Briefly, PCR was performed with 50 ng of genomic DNA, 200 µM dNTP, 2 µL ×10 PCR buffer, 1.5 mM $MgCl_2$ and 0.5 µL (3U) of Taq DNA Polymerase (AmpliTaq, Perkin Elmer, Branchburg, USA) at a final reaction volume of 20 µl.

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 2% agarose gel containing 0.5 µg/mL ethidium bromide. Amplified products were visualized under ultraviolet transilluminator (BIORAD, Diamed GmbH, Switzerland), and the results were documented.

For providing the blood transfusion therapy, five cases were selected from those patients who had positive DAT and who required packet RBC (PRBC) transfusion due to the severity of anemia. ABO Rh (D)-matched buffy coat reduced PRBC units were selected randomly, and serologic minor blood group typing for Kell, Kidd, and Duffy antigen was performed. Crossmatch testing of patients' samples was performed with donor blood units which were found to be identical for Kell, Kidd, and

Duffy antigens based on the results of molecular typing of the patient. A single episode of transfusion was given to all five patients with matched donor units. Pre- and post-transfusion laboratory parameters were compared for each patient, and posttransfusion monitoring was done to record any adverse event.

Statistical analysis

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

Results

In this study, the phenotypes as predicted by molecular genotyping were compared with the serological phenotype for Kell, Kidd, and Duffy blood group antigen in 50 normal blood donors (control) and 30 patients with AIHA.

We have observed concordance in the results of phenotype and genotype of the blood donors for all the blood group antigens studied [Table 1].

Laboratory workup of patients' samples showed DAT positivity in all 30 samples with grade of agglutination ranging from 3+ to 4+. On IAT, 22 samples (73.3%) showed panreactivity while testing with screening cell panel, with grades of reaction from 3+ to 4+. In rest of the eight samples (26.7%) which were indirect coombs test (ICT) negative, elution was performed on DAT-positive red cells using cold-acid elution procedure. Eluates from all samples were found to be panreactive while testing with screening cell panels, with grades of reaction from 2+ to 4+.

Results of serological phenotyping of minor red cell antigens showed varied degree of agglutination in all samples for all antigens tested except for K where 20 (67%) samples showed negative reaction [Table 2]. The molecular typing was able to detect the antigen frequency accurately in all the samples as described in Table 2. Upon performing the synergistic analysis, the incidence of minor blood group genotyping frequencies among AIHA patients (n = 30) is as shown in Table 3.

The pretransfusion hematological parameters (hemoglobin, reticulocyte count, serum lactate dehydrogenase [LDH], and serum bilirubin) were recorded for five patients who were transfused PRBC units which were genotypically matched for Kell, Kidd, and Duffy antigens. The cross-match testing of four units with patients' serum showed incompatibility. After the transfusion, the hematological parameters showed an improvement in the existing anemia [Table 4]. No adverse transfusion reaction was reported in any of the patients.

Table 1: Phenotyping and genotyping results for Kell, Kidd, and Duffy system antigens on samples from 50 normal blood donors

Genotype	Phenotypes		
Kell system	**K+k–**	**K–k+**	**K+k+**
K/k	0	0	2
K/K	1	0	0
k/k	0	47	0
Kidd system	**Jk (a+b–)**	**Jk (a+b+)**	**Jk (a–b+)**
Jk^a/Jk^a	18	0	0
Jk^a/Jk^b	0	20	0
Jk^b/Jk^b	0	0	12
Duffy system	**Fy (a+b–)**	**Fy (a+b+)**	**Fy (a–b+)**
Fy^a/Fy^a	24	0	0
Fy^a/Fy^b	0	19	0
Fy^b/Fy^b	0	0	7

Table 2: Phenotyping and genotyping results for Kell, Kidd, and Duffy systems on samples from 30 autoimmune hemolytic anemia patients

Minor blood group	Antigen	Serological grade of reaction	Frequency (%)	Molecular typing, frequency (%)
Kell	K	3+–4+	10 (33)	1 (3.3)
		Negative	20 (67)	
	k	2+–4+	30 (100)	30 (100)
Kidd	Jk^a	3+–4+	30 (100)	21 (70)
	Jk^b	2+–3+	30 (100)	23 (76.6)
Duffy	Fy^a	2+–4+	30 (100)	21 (70)
	Fy^b	3+–4+	30 (100)	16 (53.3)

Table 3: Incidence of minor blood group genotyping frequencies in autoimmune hemolytic anemia patients (n=30)

Gene	Genotype	Frequency (%)
Kell	K/k	1 (3)
	K/K	0
	k/k	29 (97)
Kidd	Jk^a/Jk^b	14 (47)
	Jk^a/Jk^a	7 (23)
	Jk^b/Jk^b	9 (30)
Duffy	Fy^a/Fy^b	7 (23)
	Fy^a/Fy^a	14 (47)
	Fy^b/Fy^b	9 (30)

Discussion

This study was conducted to determine the importance of performing molecular analysis for the determination of blood groups in transfusion-dependent AIHA patients. Patients with AIHA often have severe anemia and require frequent blood transfusions. Thus, they are highly susceptible to alloimmunization, with a prevalence reported ranging from 15% to 40% (mean

Table 4: Characteristics of donor units transfused and their effect on hematological parameters of autoimmune hemolytic anemia patients

AIHA case	Molecular typing based inference of phenotype	ABO Rh (D) matched random unit crossmatch	ABO Rh (D) and antigen matched unit crossmatch	Pretransfusion Hb (g/dL)	Posttransfusion Hb (g/dL)
1	K–k+Jk^a+Jk^b–Fy^a–Fy^b+	Incompatible	Incompatible	5.7	6.9
2	K–k+Jk^a+Jk^b–Fy^a–Fy^b+	Incompatible	Incompatible	5.9	7.4
3	K–k+Jk^a+Jk^b–Fy^a+Fy^b–	Incompatible	Incompatible	4.7	5.8
4	K–k+Jk^a–Jk^b+Fy^a+Fy^b–	Incompatible	Incompatible	6.2	7.6
5	K–k+Jk^a+Jk^b+Fy^a–Fy^b+	Incompatible	Compatible	4.9	6.3

AIHA case	Pretransfusion retic count (%)	Posttransfusion retic count (%)	Pretransfusion serum bilirubin (mg/dL)	Posttransfusion serum bilirubin (mg/dL)	Pretransfusion serum LDH (IU/L)	Posttransfusion serum LDH (IU/L)
1	4.6	4.4	1.7	1.5	2903	2867
2	6.3	5.3	2.1	2.4	1634	1674
3	20.7	21.4	2.4	2.8	2870	2856
4	7.1	6.3	3.1	3.2	1769	1715
5	6.6	4.9	4.7	4.9	2774	2798

Hb=Hemoglobin, AIHA=Autoimmune hemolytic anemia, LDH=Lactate dehydrogenase

32%).[6] The presence of autoantibodies in the serum masks the presence of alloantibody, potentially resulting in hemolytic transfusion reaction. Finding compatible blood is further complicated by panreactive results on IAT due to serum autoantibodies.

Several approaches have been described for the selection of RBCs for transfusion to patients. One approach is testing patient's diluted serum against a red cell panel. If a weakly reactive autoantibody and a strongly reactive alloantibody are present, the differences in the strength of the reaction of various cells of the panel will be indicative of the alloantibody. The technique is unreliable because there is no surety that alloantibody will react more strongly than autoantibody.[7] Another approach is selection of least compatible unit; wherein, a number of ABO-matched units are subjected to testing reactivity of patient's autoantibody and the unit that reacts least strongly is selected for transfusion to the patient. Das *et al*. have managed 14 patients of AIHA with severe anemia using PRBC units whose reaction strength was found less than that of the autocontrol strength, designated as "best match" or "least incompatible" units and have reported uneventful transfusion in most of the cases.[8] In another study by Park *et al*. transfused, 161 AIHA patients with least incompatible PRBC and monitored the patients over 7 days. The authors have found no increase in hemolysis risk when compared with posttransfusion patients positive for alloantibodies or those lacking RBC-specific antibodies.[9] However, this procedure has not been considered an acceptable alternative for selecting donor units for the transfusion of patients with AIHA in the modern-day transfusion practice except in extremely urgent settings, in which there is not time to perform adequate serologic tests.[10]

The most popular approach is performing adsorption test for removing autoantibody from patients' sera and thereafter performing compatibility testing with adsorbed sera. The technique is very lengthy (takes 4–6 h) and is often rendered even more cumbersome and less accurate if the patient has been transfused in the recent past because even a small percentage of transfused cells may adsorb the alloantibody during the *in vitro* adsorption procedure, thus invalidating the results. Moreover, severe anemia in patients may preclude obtaining a large enough volume of RBCs for the autoadsorption procedure.[11]

Thus, transfusion of prophylactic antigen-matched RBC has been suggested by Shirey *et al*., to avoid and replace complex adsorption testing.[12] The authors have concluded that the algorithm for providing prophylactic antigen-matched RBCs to these patients when a complete phenotype can be determined provides flexibility in their transfusion management while maintaining safety and circumvents or simplifies pretransfusion adsorption studies. However, it is not possible to determine the serological blood group phenotype accurately when hemagglutination is performed on the samples of these patients when the red cells are strongly DAT positive or if the patient has been recently transfused. Molecular typing is increasingly used as a valuable tool to determine the correct blood group phenotype in such cases so that patients may receive appropriate transfusion support with reduced risk of transfusion reactions and alloimmunization.

We have determined both phenotype and genotype for Kell, Kidd, and Duffy antigens in normal blood donors using SSP-PCR. The results were found to be in concordance in 100% cases indicating that molecular genotyping protocols can be effectively applied to the North Indian ethnic population. Serological typing for Kell, Kidd, and Duffy antigens in AIHA patients showed inconclusive results due to DAT positivity.

Molecular typing of patient samples led to clear-cut determination of Kell, Kidd, and Duffy phenotypes. The patients' samples were subjected to crossmatch with random ABO-matched PRBC units and incompatibility was seen in all five cases. Further, crossmatch was done with phenotype-matched PRBC units as per the results of molecular typing. Four out of five tests showed incompatible results. However, transfusion of the patients was done using these phenotype-matched PRBC units under close supervision, and no adverse hemolytic events were reported. Further laboratory evaluation of patients' samples showed satisfactory increment in posttransfusion hemoglobin levels (mean increment of 1.32 g/dL). There was no derangement in the levels of other markers of hemolysis (reticulocyte count, serum bilirubin, and serum LDH) even after 24 h of the PRBC transfusion.

Molecular blood group typing has proved to be immensely useful in the determination of actual antigen profile and hence in providing appropriate transfusion support in patients with thalassemia and sickle cell anemia. The multitransfused patients have erroneous results on serological phenotyping due to the presence of donors' RBC cells in the circulation. Highlighting the importance of molecular blood group phenotyping, may studies have reported discrepant results on serological phenotyping in multitransfused patients. Bakanay *et al*.[13] have found discrepancies in genotype and phenotype of 51% of thalassemia and sickle cell anemia patients, Belsito *et al*.[14] have observed discordance in 54% transfusion-dependent patients, and Sarihi *et al*.[15] have reported discrepancies in 41.5% thalassemia patients. From India, Kulkarni *et al*.[16] have reported discrepant results in 77% of thalassemia patients, Shah *et al*.[17] have reported 80% variance in thalassemia and sickle cell anemia patients, and Sonker *et al*.[18] have reported discordance in 24% of thalassemia patients.

There have been very few studies in the past, in which molecular blood group phenotyping has been performed in AIHA patients. In the present study, we have found that there was varied degree of agglutination on serological phenotyping in 100% of patient samples, and the true phenotype could be determined only through molecular blood grouping. El Kenz *et al*.[19] have evaluated the feasibility of selecting antigen-matched units based on RBC genotyping for patients with AIHA. They had included RBC samples from seven patients with AIHA presenting a strongly positive DAT. Matched donor units were selected according to the genotype. Four patients received antigen-matched RBC units based on RBC genotyping. After each transfusion, the recovery was recorded and considered satisfactory for all transfused patients. Authors have found that RBC genotyping is highly beneficial for patients with AIHA and makes the selection of compatible blood considerably easier, even in an emergency context.

Conclusion

Molecular blood group typing has proved immensely useful in the determination of actual antigen profile and hence in providing appropriate transfusion support in patients with AIHA reduced risk of transfusion reactions and alloimmunization.

Despite a small sample size, the results of our study emphasize that blood group genotyping is greatly beneficial in selecting compatible blood for transfusion in AIHA patients. Although in our part of the world, laboratories capable of performing the molecular typing are present only at selected blood centers, the safety of blood transfusion is greatly enhanced if serological phenotyping is replaced with molecular techniques for reliable phenotype determination in AIHA patients requiring blood transfusion. The major limitation of our study is that we could not do genotyping for Rh antigen system and other clinically significant antigen systems such as M, N, S, and sc. The authors recommend a further study with larger sample size, clinical and laboratory correlation, and involving more antigen systems to confirm the efficacy of genotyping compared to serologic procedures in decreasing the risk of alloimmunization, delayed hemolytic transfusion reaction, and enhancing the survival of transfused red cells.

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Conflicts of interest
There are no conflicts of interest.

References

1. Petz LD, Garratty G. Acquired Immune Hemolytic Anemias. New York: Churchill Livingstone; 1980.
2. Regina ML. The positive direct antiglobulin test and immune mediated hemolysis. In: American Association of Blood Banks ed. Technical Manual. 16th ed. Bethesda, Maryland: AABB; 2008.
3. Raos M, Lukic M, Pulanic D, Vodanovic M, Cepulic BG. The role of serological and molecular testing in the diagnostics and transfusion treatment of autoimmune haemolytic anaemia. Blood Transfus 2022;20:319-28.
4. Daniel J. Sampling Essentials: Practical Guidelines for Making Sampling. New Delhi: SAGE Publications India Pvt. Ltd; 2012.
5. Judd WJ, Johnson ST, Storry J. Judd's Methods in Immunohematology. 3rd ed. Bethesda, MD: AABB Press; 2008.
6. Branch DR, Petz LD. Detecting alloantibodies in patients with autoantibodies. Transfusion 1999;39:6-10.
7. Leger RM, Garratty G. Evaluation of methods for detecting alloantibodies underlying warm autoantibodies. Transfusion 1999;39:11-6.
8. Das SS, Zaman RU, Safi M. Incompatible blood transfusion: Challenging yet lifesaving in the management of

acute severe autoimmune hemolytic anemia. Asian J Transfus Sci 2014;8:105-8.
9. Park SH, Choe WH, Kwon SW. Red blood cell transfusion in patients with autoantibodies: Is it effective and safe without increasing hemolysis risk? Ann Lab Med 2015;35:436-44.
10. Petz LD. A physician's guide to transfusion in autoimmune haemolytic anaemia. Br J Haematol 2004;124:712-6.
11. Laine EP, Leger RM, Arndt PA, Calhoun L, Garratty G, Petz LD. *In vitro* studies of the impact of transfusion on the detection of alloantibodies after autoadsorption. Transfusion 2000;40:1384-7.
12. Shirey RS, Boyd JS, Parwani AV, Tanz WS, Ness PM, King KE. Prophylactic antigen-matched donor blood for patients with warm autoantibodies: An algorithm for transfusion management. Transfusion 2002;42:1435-41.
13. Bakanay SM, Ozturk A, Ileri T, Ince E, Yavasoglu S, Akar N, *et al.* Blood group genotyping in multi-transfused patients. Transfus Apher Sci 2013;48:257-61.
14. Belsito A, Costa D, Fiorito C, De Iorio G, Casamassimi A, Perrotta S, *et al.* Erythrocyte genotyping for transfusion-dependent patients at the azienda universitaria policlinico of Naples. Transfus Apher Sci 2015;52:72-7.
15. Sarihi R, Oodi A, Dadkhah Tehrani R, Jalali SF, Mardani F, Azarkeivan A, *et al.* Blood group genotyping in alloimmunized multi-transfused thalassemia patients from Iran. Mol Genet Genomic Med 2021;9:e1701.
16. Kulkarni S, Choudhary B, Gogri H, Patil S, Manglani M, Sharma R, *et al.* Molecular genotyping of clinically important blood group antigens in patients with thalassaemia. Indian J Med Res 2018;148:713-20.
17. Shah A, Patel P, Patel K, Patel B, Jariwala K, Sharma P, *et al.* Comparison of serology and molecular detection of common red cell antigens in multitransfused thalassemia major and sickle cell disease patients. Transfus Apher Sci 2020;59:102599.
18. 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.
19. El Kenz H, Efira A, Le PQ, Thiry C, Valsamis J, Azerad MA, *et al.* Transfusion support of autoimmune hemolytic anemia: How could the blood group genotyping help? Transl Res 2014;163:36-42.