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Comparison of Blood Group Molecular Genotyping to Traditional Serological Phenotyping in Patients with Chronic or Recent Blood Transfusion

Zhan Ye[1], Daoping Zhang[1], Leonard Boral[1,2], Counts Liz[2], John May[1]

[1]Department of Pathology and Laboratory Medicine, University of Kentucky, Lexington, KY, USA
[2]Kentucky Blood Center, 3121 Beaumont Centre Circle, Lexington, KY, USA
Email: zye2@uky.edu

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Abstract

Objectives: Accurately identifying the Antigens (Ags) on recipient red blood cells (RBCs) is critical in prevention of RBC alloimmunization in chronically transfused patients. The goal of this study was to compare RBC molecular genotyping to serological phenotyping in those patients. Methods: Serological phenotyping and molecular genotyping methods were used to study blood samples from 18 healthy blood donors and 16 transfused patients. Reticulocyte harvesting or hypotonic cell separation was added to recheck RBC phenotypes of the patients with discrepancies between phenotyping and genotyping. Results: No discrepancies were found between the two genotyping methods in all the donors and patients. 1 of 9 sickle-cell disease (SCD) patients and all 3 thalassemia patients demonstrated discrepancies in multiple blood groups between phenotyping and genotyping, which were not corrected by reticulocyte harvesting or hypotonic cell separation. Conclusions: These findings suggest that RBC molecular genotyping is superior to serological phenotyping in chronically transfused SCD or thalassemia patients.

Keywords

Blood Group, Molecular Genotyping, Serological Phenotyping, Chronic Blood Transfusion

1. Introduction

SCD is an autosomal recessive genetic blood disorder characterized by red cells which transform into abnormal, rigid, sickle shaped red RBCs, when they are in a hypoxic environment. SCD may lead to various acute complications with a high mortality rate such as splenic sequestration crisis, aplastic crisis and hemolytic crisis [1]. Blood transfusion with normal hemoglobin A RBCs is thought to increase oxygen saturation, and reduce red blood cell sickling. Therefore, blood transfusion is the effective and common management of acute complications of SCD [2]. Furthermore, chronic RBC transfusion therapy has been shown to be efficacious in reducing

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the risk of stroke or silent stroke in children with SCD [3]. Thalassemia is another inherited autosomal recessive blood disorder caused by the increased destruction of red blood cells due to defects in either the α or β chain in hemoglobin. Chronic anemia in these patients may also cause extramedullary hematopoiesis, which can lead to bone deformities. These patients may need life-long RBC transfusions to ameliorate the chronic anemia and further suppress extramedullary hematopoiesis.

One of the major side effects of chronic blood transfusion therapy is alloimmunization to foreign RBCs. The incidence of RBC alloimmunization in general population is approximately 2% to 6%, however, it may be as high as 36% in patients with SCD [4]. Alloimmunization is the source of a variety of problems during long-term RBC transfusion management, and is associated with an increased incidence of delayed hemolytic transfusion reactions and reduction in available compatible blood for future transfusion in subsequent crisis [5].

There are at least 22 blood group systems, among which ABO, Rh, Kell, Duffy, and Kidd blood groups contain the Ags provoking the most severe transfusion reactions. Whenever possible, these chronic blood transfusion recipients whose RBCs lack those Ags should receive compatible Ag-negative RBCs to avoid generation of corresponding antibodies. Therefore, accurately identifying the Ags on recipient RBCs is critical in prevention of RBC alloimmunization.

However, due to the presence of donor RBCs in the recipients' circulation which may persist for months, accurate Ag typing of these transfused recipients by traditional serological methods is often a difficult if not impossible task. Furthermore, Ag typing is more complicated when the recipient's RBCs have a positive direct antiglobulin test.

For recently transfused patients who need Ag typing it is possible to isolate the patient's RBCs from the transfused donor RBCs by cell separation techniques. In the capillary tube method the recipient's larger and lighter reticulocytes will be present near the plasma interface after centrifugation while the heavier older RBC that are transfused will appear in the deepest layers of the red cells. A file can be used to score the capillary tube a few millimeters below the plasma interface and into the RBC layer. The capillary tube can then be snapped apart and the presumed lighter small reticulocyte layer (from the recipient) with the plasma can be washed and the RBCs derived can be tested for red cell Ags. This technique cannot be used in patients who are receiving chemotherapy or who have bone marrow failure because reticulocytes from the patient will not be generated in sufficient numbers for testing and instead, donor RBC may replace the patient reticulocytes in this layer. A second technique used exclusively in SCD patients can separate Hgb S RBCs (patient) from Hgb A RBC (donor) by exposing this mixture to a hypotonic solution. In this system, the donor hemoglobin A RBCs will lysed while the resistant Hgb S RBCs will remain and be available for RBC Ag typing after washing them.

With the rapid progression of DNA technology our understanding of the molecular basis of many blood group Ags has increased and made genotyping of RBC Ags possible. One major concern of genotyping recipient blood is the contamination of recipient DNA by the DNA from donor WBCs. However, studies have shown that the donor's DNA does not interfere in the process because the amount of recipient's DNA is far greater than DNA from donor's WBCs [6].

Previous studies on chronically transfused patients with SCD or thalassemia showed discrepancies between the serologic RBC phenotype and phenotype predicted by the genotype in the patients receiving chronic blood transfusion but not in the blood donor group [7] [8]. However the specificity of DNA sequences detection in these studies was based on the size of PCR products and RFLP analysis which needs overnight digestion. More accurate and rapid methods are required for real time patient care.

The majority of blood group polymorphisms are associated with a single point mutation or single nucleotide polymorphisms (SNPs) in the gene encoding the protein carrying the blood group Ag or the enzymes catalyzing the addition of monosaccharide onto a nascent blood group oligosaccharide moiety (*i.e*. ABO system) [9]. One well known example is K/k (K1/K2) polymorphism, where a SNP in exon 6 (codon 193) of the gene encoding the Kell blood group (KEL) is altered from Met (K) to Thr (k) [10]. The clear relationship between SNPs of RBC genes and RBC phenotypes makes it possible to overcome the limitations of the serological methods and provide precise RBC Ag determination of a patient's phenotype possible in the presence of transfused donor RBC.

Several new RBC genotyping assays based on SNPs detection have been developed recently. By using high specific primers for SNPs of genes encoding RBC Ags and high sensitive detection techniques, e.g. Luminex, these assays can identify the genotype of RBCs in few hours. In this study, we compared commercial RBC ge-

notyping assays and the traditional test tube hemagglutination assay for RBC Ags in the management of SCD or thalassemia patients receiving chronic blood transfusion therapy. We studied blood samples from 18 healthy blood donors, as well as 16 patients including 9 SCD, 3 thalassemia patients and 4 (transfused) patients without SCD or thalassemia by genotyping them for assays yielding RBC Ags in the MNS, Rh, Kell, Duffy, and Kidd systems. Discrepancy between test tube hemagglutination method and genotyping assays were found in the specimen of 4 patients (4/16) and 1 blood donor (1/18). Among patients found discrepancy, there were 3 thalassemia patients and 1 SCD patient. Samples with discrepancies were tested with alternative molecular method to confirm the RBC Ags. The results were consistent among different molecular assays. However, variations of antigen typing were found in serological testing when the RBC Ags were retyped by the same serological method in the same facility, or in another facility; or a cell separation technique was added before typing. Our study indicated RBC Ags typing by serological tube hemagglutination method could be affected by multiple factors, such as recent transfusion, technologist variance, and patient's current clinical conditions which might affect harvesting autologous red cells from transfused donor red cells. These observations suggested that molecular genotyping assays are more accurate than the serological test tube hemagglutination method for patient with recent transfusion.

2. Materials and Methods

2.1. Patients and Blood Donors

We studied peripheral blood samples from 16 patients including 9 with SCD, 3 with thalassemia and 4 without SCD or thalassemia harvested at Blood Bank, University of Kentucky Medical Center (UKMC). 18 blood donor samples were provided by the Kentucky Blood Center (KBC). The donor samples were from healthy individual without recent transfusion.

2.2. Test Tube Hemagglutination Method (TUBE) for RBC Ags Identification (Traditional Serological Phenotyping)

For all the donors and patients, RBC phenotypes were determined by TUBE using commercial sources of anti-sera (Immucor or Biorad) at KBC.

For 2 thalassemia patients with discrepancies between TUBE and molecular genotyping, reticulocytes (newly formed autologous red cells) were harvested by microhematocrit centrifugation [11] and phenotyped by TUBE. Since newly formed autologous red cells (reticulocytes or neocytes) generally have a lower specific gravity than transfused red cells, the reticulocyts migrate to the top of the column of red cells when blood is centrifuged in microhematocrit tubes. The harvested reticulocytes were phenotyped by the same protocol as donor RBCs at KBC.

For 1 SCD patient with discrepancies between TUBE and molecular genotyping, sickle cells from the same sample were harvested by hypotonic cell separation technique (11) and rechecked by TUBE. In brief, the technique is performed by washing cells 6 times with 0.3% NaCl followed by 2 washes with normal saline. Sickle cells (HbSS or HbSC) are resistant to lysis by hypotonic saline, in contrast to red cells from normal persons (HbAA) and those with hemoglobin S trait (HbAS). This procedure permits isolation of autologous red cells from patients with sickle cell disease who have recently been transfused.

2.3. Genotyping RBC Ags by Two Different Commercial Kits

(1) LIFECODES RBC:

We used LIFECODES RBC (GEN-PROBE, San Diego, CA) for genotyping our RBCs. The kit detects highly prevalent SNPs of six blood group Ags through the Polymerase Chain Reaction – Reverse Sequence Specific Oligonucleotide (PCR-RSSO) method. The blood groups studied in this paper were Kell (K, k, Kpa, Kpb, Kpc, Jsa, Jsb), Kidd (Jka, Jkb, Jk (Finnish null)), Duffy (Fya, Fyb, Fyx (a-b weak), FyGATAsil), MNS (M, N, S, s, S-s-Uvar (includes 2 S silencing mutations: −GPB230 and GPB intron +5)), Rh (C, c, E, e). PCR products were detected using Luminex technology.

DNA from peripheral blood (white blood cells) was extracted by QIAGEN EZ1 (QIAGEN Inc.). PCR analysis followed the protocol provided by GEN-PROBE and data was analyzed using LIFECODES MATCH IT!-RBC software.

The PCR reaction and detection takes about 4 hours and 1 hour, respectively. 96 samples can be analyzed in one reaction.

LIFECODES RBC was the primary method used to detect the RBC genotypes for all the blood samples. This test was performed in the Immunomolecular Pathology (IMP) lab at UKMC.

(2) RED CELL EZ TYPE (GEN-PROBE, Inc., San Diego), performed by Gen-PROBE in selected situations:

RED CELL EZ TYPE assay is based on PCR-SSP (sequence-specific priming) technique. PCR-SSP requires a number of amplifications carried out in parallel to amplify only the alleles present in the template. In each PCR tube, Human Growth Hormone gene is used as internal control. PCR products stained by ethidium bromide are separated by agrose gel electrophoresis on pre-cast E-Gels and detect on an UV transilluminator.

This kit was used as the second genotyping method when discrepancy between TUBE and LIFECODESRBC assay were found

3. Result

3.1. Serological Phenotyping and Molecular Genotyping in Donor Blood Samples

The comparison between TUBE and molecular genotyping methods for all five RBC Ag groups in donor blood samples are listed in **Table 1**.

For the 18 donor blood samples, the results from serological phenotyping and molecular genotyping were the same except for 1 donor with discrepancy in typing of E (**Table 2**). This sample was E negative by TUBE but E positive by LIFECODES RBC and confirmed as E positive with EZ TYPE.

3.2. Serological Phenotyping and Molecular Genotyping in 9 SCD Patients

The comparison between TUBE and molecular genotyping methods for all five RBC Ag groups in SCD patients are listed in **Table 3**.

8 of 9 SCD patients show identical results of all five Ag groups from serological phenotyping and molecular genotyping. However, one patient who was recently transfused has several discrepancies for three Ags: S, Fy(a), Jk(b) (**Table 4**). Hypotonic cell separation was applied to this sample and sickle cells were harvested in both UKMC and KBC. The Ags showing discrepancy were rechecked by TUBE. The molecular genotyping results were repeatable with different molecular methods at different labs (LIFECODES RBC at IMP-UKMC and RED

Table 1. Comparison between TUBE and molecular genotyping in blood donors.

	Identical/total[1]	Discrepancy/total[2]
MNS	18/18	0/18
RhDCe	17/18	1/18 (See E Ag typing on **Table 2**)
Kell	18/18	0/18
Duffy	18/18	0/18
Kidd	18/18	0/18

1. identical/total = number of donors with identical results from TUBE and molecular genotyping/number of total blood donors; 2. discrepancy/total = number of donors with discrepancy between TUBE result and molecular genotyping result/number of total blood donors.

Table 2. Discrepancy in one donor between TUBE and molecular genotyping result. The Ag group showing discrepancy is highlighted.

	RhDCe			
	c	C	e	E
TUBE	+	+	+	-
LIFECODES RBC	+	+	+	+
RED CELL EZ TYPE	+	+	+	+

Table 3. Comparison between TUBE and molecular genotyping in 9 SCD patients.

	Identical/total[1]	Discrepancy/total[2]
MNS	8/9	1/9 (See S Ag typing on **Table 4**)
RhDCe	9/9	0/9
Kell	9/9	0/9
Duffy	8/9	1/9 (See Fy(a) Ag typing on **Table 4**)
Kidd	8/9	1/9 (See Jk(b) Ag typing on **Table 4)**

1. identical/total = number of SCD patients with identical results from TUBE and molecular genotyping/number of total SCD patients; 2. discrepancy/total = number of SCD patients with discrepancy between TUBE result and molecular genotyping result/number of total SCD patients.

Table 4. Discrepancy in one SCD patient among TUBE, TUBE plus hypotonic cell separation and molecular genotyping results. The Ag groups showing discrepancy are highlighted.

	MNS				Duffy		Kidd	
	M	N	S	s	Fy(a)	Fy(b)	Jk(a)	Jk(b)
LIFECODES RBC	+	+	+	+	-	-	+	-
RED CELL EZ TYPE	+	+	+	+	-	-	+	-
TUBE	+	+	-	+	+	-	+	+
UKMC-Sickle cells[1]			-		-			-
KBC-Sickle cells[2]			-		-			+

1. In UKMC, the sample was treated with hypotonic solution to harvest patient autologous red cells (sickle cells) and then rechecked by TUBE. 2. In KBC, the sample was treated with hypotonic solution to harvest patient autologous red cells (sickle cells) and then rechecked by TUBE.

CELL EZ TYPE at GEN-PROBE). However, some of the serological phenotyping results varied from molecular genotyping results. At both UKMC and KBC, the S Ag typing was negative whether using the unaltered RBCs or hypotonic wash, while both molecular techniques showed the S Ag to be positive. With the unaltered RBC TUBE test, the Fy(a) was positive at both UKMC and KBC. The hypotonic cell wash, however, resulted in negative in Fy(a) typing. This negative Fy(a) Ag typing was also seen in both molecular tests. In the unaltered RBC TUBE test technique, Jk(b) Ag testing was positive. The hypotonic cell wash technique showed variable results that the Jk(b) Ag typing was negative at UKMC, but positive at KBC. Interestingly, both molecular methods were negative for Jk(b) typing.

3.3. Serological Phenotyping and Molecular Genotyping in 3 Thalassemia (T) Patients

Reticulocytes were harvested from 3 multiply, recently transfused, T patients (arbitrarily named as T1, T2 and T3). All of these patients received transfusion chronically. Antigen typing discrepancies were found in all 3 patients and in 6 of the Ags (M, c, E, K, Jk(a) and Jk(b)) (**Table 5**). Jk(a) had the highest frequency of discrepancy which was present in all three patients. Jk(b) and M showed discrepancies in two patients, while one discrepancy was noted in each of K, c and E Ags (**Table 6**).

For patients with discrepancies between serological phenotyping and molecular genotyping, the results of molecular genotyping were identical using 2 different kits (LIFECODES RBC and RED CELL EZ TYPE, data not shown).

Reticulocytes were harvested in 2 thalassemia patients (T1 and T2) at both UKMC and KBC (**Table 7**). Their Ag expression, original detected by TUBE using unaltered RBCs, was rechecked by TUBE using reticulocytes which showed varied serological phenotyping (probable interference by donor RBCs) (**Table 7**). Discrepancies were found between TUBE using unaltered RBC and TUBE using reticulocytes (T1-c, T1-K, T1-Jk(a) and T2-E). Comparison between TUBE using reticulocytes and molecular genotyping also showed discrepancies in c Ag of T1, as well as M, c Jk(a) and Jk(b) Ags of T2. Those discrepancies were probably due to very low reticulocyte counts in thalassemia patients, and therefore low number of patients' own RBCs.

Table 5. Comparison between TUBE and molecular genotyping in T patients.

	Identical/total[1]	Discrepancy/total[2]
MNS	1/3	2/3 (See M Ag typing on **Table 6**)
RhDCe	1/3	2/3 (See E,c Ag typing on **Table 6**)
Kell	2/3	1/3 (See K Ag typing on **Table 6**)
Duffy	3/3	0/3
Kidd	0/3	3/3 (See Jk(a) and Jk(b) Ag typing on **Table 6**)

1. identical/total = number of T patients with identical results from TUBE and molecular genotyping/number of total T patients; 2. discrepancy/total = number of T patients with discrepancy between TUBE result and molecular genotyping result/number of total T patients.

Table 6. Discrepancy in 3 T patients between TUBE and molecular genotyping result. The Ag groups showing discrepancy are highlighted.

		MNS				RhDCe			Kell			Kidd	
		M	N	S	s	c	C	e	E	K	k	Jk(a)	Jk(b)
T1	TUBE, unaltered[1]	+	+	-	+	+	+	+	-	+	+	+	+
	Molecular[2]	+	+	-	+	+	+	+	-	-	+	-	+
T2	TUBE, unaltered	+	+	-		+	+	+	+	-	+	+	-
	Molecular	-	+	-	+	-	+	+	-	-	+	-	+
T3	TUBE, unaltered	+	+	-	+	+	-	+	-	-	+	-	+
	Molecular	-	+	-	+	+	-	+	-	-	+	+	-

1. TUBE, unaltered: TUBE using unaltered RBCs. 2. Molecular: molecular genotyping.

Table 7. Comparisons of results among TUBE using unaltered RBCs, molecular genotyping and TUBE using reticulocytes. Only Ag groups showing discrepancies are listed.

		MNS	RhDCe		Kell	Kidd	
		M	c	E	K	Jk(a)	Jk(b)
T1	TUBE, unaltered[1]		+		+	+	
	UKMC reticulocytes[2]		NA[3]		-	-	
	KBC reticulocytes[4]		-		-	-	
	Molecular[5]		+		-	-	
T2	TUBE, unaltered	+	+	+		+	-
	UKMC reticulocytes	+	+	-		+	-
	KBC reticulocytes	NA	+	-		+	-
	Molecular	-	-	-		-	+

1. TUBE, unaltered: TUBE using unaltered RBCs; 2. RBC phenotyping of reticulocytes harvested by cell separation technique at UKBC; 3. NA: not applicable; 4. RBC phenotyping of reticulocytes harvested by cell separation technique at KBC; 5. Molecular: molecular genotyping.

3.4. Serological Phenotyping and Molecular Genotyping in Recently Transfused Non-SCD or Non-Thalassemia (RTnSnT) Patients

All 4 RTnSnT patients received recent blood transfusion within 3 months before tests. None of them showed discrepancy between serological and molecular typing (**Table 8**). The results of molecular genotyping were identical using different molecular assays (LIFECODES RBC and RED CELL EZ TYPE, data not shown).

Table 8. Comparison between TUBE and molecular genotyping in RTnSnT patients.

	Identical/total[1]	Discrepancy/total[2]
MNS	4/4	0/4
RhDCe	4/4	0/4
Kell	4/4	0/4
Duffy	4/4	0/4
Kidd	4/4	0/4

1. identical/total = number of RTnS patients with identical results from TUBE and molecular genotyping/number of total RTnS patients; 2. discrepancy/total = number of RTnS patients with discrepancy between TUBE result and molecular genotyping result/number of total RTnS patients.

4. Discussion

For the recipients of chronic transfusion, the major obstacle to get the accurate RBC phenotype by TUBE is the contamination by donor RBCs. Therefore, several techniques were used to solve this problem. The principle is either to harvest the autologous red cells by separating the recipient RBCs from donor cells or destroy the donor cells.

For patients with Thalathessmia or RTnSnT patients, isolation of autologus red cells (reticulocytes) from the blood sample by capillary tube method is critical for accuracy of serological phenotyping. In the capillary tube method the recipient's larger and lighter reticulocytes will be present near the plasma interface after centrifugation while the heavier spherocyte-like older RBC that are transfused will appear in the deepest layer of the red cells. A file can be used to score the capillary tube a few millimeters below the plasma interface and into the RBC layer. The capillary tube can then be snapped apart and the presumed lighter small reticulocyte layer (from the recipient) with the plasma can be washed and the RBCs derived can be tested for red cell Ags.

The purity of reticulocytes harvested by the capillary tube method is significantly affected by the ratio of recipient reticulocytes/donor RBCs. However, in chronic transfusion dependent patients, this ratio is often low because usually very few reticulocytes are made for the following possible reasons.

Frequently infused exogenous RBCs inhibit the endogenous erythropoiesis, probably due to decrease in erythropoietin (EPO) production. EPO is a glycoprotein hormone that promotes the survival, proliferation and differentiation of erythrocyte precursors in the bone marrow. The major stimulator for EPO synthesis is hypoxia [12]. In Thalassemia patients, the hypoxia caused by long term anemia could stimulate EPO production which in turn supports endogenous erythropoiesis. However, chronic transfusion corrects anemia and stops EPO production.

EPO is produced by interstitial fibroblasts in the kidney and perisinusoidal cells in the liver. While liver production predominates in the fetal and perinatal period, renal production is predominant during adulthood [13]. For the patients with chronic renal failure and cirrhosis (the RTnSnT patient in **Table 8**), the decreased percentage of endogenous reticulocytes could result from lack of EPO.

Furthermore, for patients receiving chemotherapy, or having bone marrow failure or inefficient erythropoiesis, donor RBCs may replace the patient reticulocytes layer in capillary tube because endogenous reticulocytes will not be generated in sufficient numbers for testing. Thalassemia patients have the combination of inefficient erythropoiesis and chronic transfusion, which could explain the high frequency of discrepancies between TUBE-reticulocytes and TUBE-unaltered RBCs, as well as between TUBE-reticulocytes and molecular genotyping of these patients. Therefore, molecular genotyping is probably the only reliable method to identify the RBC Ags of those patients.

SCD patients also have a similar combination of chronic anemia and multiple transfusion as Thalassemia patients so alternative technique is necessary for these patients. Red cells from patients with sickle cell disease (HbSS) are theoretically more resistant to lysis by hypotonic saline than red cells from normal persons (HbAA) or those with hemoglobin S trait (HbAS). By using this inherent resistance of sickle cells to osmotic stress, patient red cells (HbSS) can be rapidly isolated from donor red cells (HbAA or HbAS) by washing with hypotonic (0.3%) NaCl [14]. However, this technique did not work well in our hands. Perhaps for SCD patients who are largely transfusion dependent (the patient in **Table 4**), hypotonic saline wash cannot generate enough patients' own RBCs to be recognized in TUBE RBC Ag typing. In that condition, molecular phenotyping is apparently

more accurate and time-efficient method than hypotonic cell separation technique plus TUBE.

In summary, this study highlights the consistency of RBC molecular genotyping in patients who are multiply transfused compared to TUBE hypotonic cell wash (in SCD patients) and reticulocyte harvesting technique (in thalassemia patients) which show variability and inconsistency. The reasons for these discrepancies is most likely due to increased hematocrit from transfusion with the subsequent decrease of EPO and reticulocytes, as well as decrease in production of patients' own RBCs. Only a few cases were studied in current study. Therefore, it is only a pilot work for this topic. Further work will be performed to fortify our findings.

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