Benefits of Blood Group Genotyping in Multi-Transfused Patients from the South of Brazil

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We evaluated the usefulness of blood group genotyping as a supplement to hemagglutination to determine the red blood cell (RBC) antigen profile of polytransfused patients with hematological diseases and renal failure. Seventy-nine patients were selected. They all received more than three units of blood and eight (10%) had already clinical significant alloantibodies occurring alone or in combination against Rh, K, Fya, and Di antigens. DNA was prepared from blood samples and *RHCE*E/e*, *KEL*01/KEL*02*, *FY*01/FY*02* and *JK*01/JK*02* alleles were determined by using PCR-RFLP. *RHD*/RHD*Ψ* and *RHCE*C/c* were tested

using multiplex PCR. Discrepancies for Rh, Kell, Duffy, and Kidd systems were found between the phenotype and genotype-derived phenotype in 16 of the 38 chronically transfused patients. The genotypes of these patients were confirmed by DNA array analysis (HEA Beadchip™; Bioarray Solutions, Warren, NJ). Genotyping was very important for the determination of the true blood groups of the polytransfused patients, helped in the identification of suspected alloantibodies and in the selection of antigen-negative RBCs for transfusion. J. Clin. Lab. Anal. 24:311–316, 2010. © 2010 Wiley-Liss, Inc.

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INTRODUCTION

Programs to prevent alloimmunization to red blood cell (RBC) antigens have been designed and implemented to provide antigen-matched RBC transfusions to patients who are alloimmunized and/or in need of chronic transfusion support (1–5). This is usually carried out by classical serology techniques that can also identify alloantibodies. However, accurate phenotyping of polytransfused patients is often complicated either by the presence of transfused donor RBCs in the recipient's circulation and by positive direct antiglobulin tests or by the lack of available direct agglutinating antibodies.

Blood group genotyping assays have been employed as an alternative for problems encountered by serology and are being used for assessing the risk of hemolytic disease in newborns (6–12). Although the use of

peripheral blood leukocytes for the genotyping of polytransfused patients has generated some concerns because of the theoretical risk of contamination of the specimen having been tested with donor leukocytes (13–16), several reports have shown that blood samples from transfused patients can be safely used for DNA typing of blood groups because the amount of DNA of

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the patient far exceeds that of contaminating donor leukocytes (9,16,17).

To achieve safe red cell transfusions, DNA analysis for minor blood group typing plays a support role in transfusion medicine, especially to provide antigen-matched blood for chronically transfused patients (18–20).

This study evaluated the contribution of DNA genotyping of RBC antigens as a tool for the management of polytransfused patients in order to overcome the limitations of hemagglutination assays and to compare the phenotype results with the genotyping carried out by PCR-RFLP in 38 polytransfused patients with different pathologies. We aimed at demonstrating the relevance of performing molecular analysis for determination of blood groups in these polytransfused patients.

MATERIAL AND METHODS

Patients

Seventy-nine patients with hematological diseases and renal failure who received multiple transfusions agreed to participate in this study by signing an IRB approved informed consent. Thirty-eight of these 79 patients were previously phenotyped for ABO, Rh (D, C, c, E, e), K/k, Fy^a/Fy^b, and Jk^a/Jk^b. All the patients had received three or more units of RBCs.

Donors

Four hundred blood donors served as controls of our procedure. They were also used to determine the genotype frequencies in this population. This control group was representative of the ethnic background of the patients.

Agglutination Tests

Phenotypes were determined by hemagglutination in gel cards after the instructions of the manufacturer (Diamed AG, Morat, Switzerland).

DNA Preparation

Genomic DNA was extracted from 200 µl aliquots of whole blood by using EZ-DNA kit (Biological Industries®; Kibbutz Beit Haemek, Israel) according to the instructions of the manufacturer and eluted into 100 µl of buffer.

PCR Amplification

Primers and amplification conditions used were the same as previously published (9,10). Briefly, PCR was performed with 100–200 ng of DNA, 50 pmoles of each

primer, 2 nmoles of each dNTP, 1.0 U *Taq* DNA polymerase, and buffer in a final volume of 50 µl.

Multiplex PCR

*RHCE**C/c genotyping was performed by a multiplex assay, which detects the presence of *RHD** intron 4 and exon 7, differentiates *RHCE**C/c, and identifies *RHD**Ψ (21). Identification of partial D was also performed by a multiplex assay, which detects several hybrid alleles (22).

RFLP Analysis

For RFLP analyses, PCR-amplified products were digested overnight with the appropriate restriction enzymes (MBI Fermentas, Amherst, NY or New England Biolab, Beverly, MA) in a final volume of 20 µl, using 10 µl of amplified product and enzyme in 1× buffer according to the instructions of the manufacturer. *MnII* enzyme was used to determine *RHCE**E/e polymorphism, whereas the enzymes *BsmI*, *MnII*, *BanI*, *StyI*, and *MspAI* were used to determine, respectively, *KEL**01/*KEL**02 (698C>T), *JK**01/*JK**02 (838A>G), *FY**01/*FY**02 (125G>A), *GATA* (–33T>C), and *FY**X (265C>T).

BeadChip DNA Analysis

The DNA array analysis was performed by using the BeadChip Human Erythrocyte Antigen (“HEA”) containing probes directed to polymorphic sites in *RHCE*, *FY* (including *FY*-*GATA* and *FY*265), *DO* (including *HY* and *JO*), *CO*, *DI*, *SC*, *GYPA*, *GYPB* (including markers permitting the identification of U-negative and U-variant types), *LU*, *KEL*, *JK*, *LW* and one mutation associated with hemoglobinopathies (BioArray Solutions) for all controls, donors, and patients samples. The HEA BeadChip assay was performed in accordance with a previously described protocol (23,24).

RESULTS

Genotype Frequencies

The genotype frequencies observed in the two studied groups (patients and blood donors) are shown in Table 1. No significant differences were observed.

Patients

Of the 79 patients selected, eight (10%) had alloantibodies. There were 13 alloantibodies, occurring alone or in combination (Table 2). One of these patients became immunized to the Di^a antigen, one to Kp^a, one to RhD, one to RhE, two to RhC and RhE, one to RhE, K, and Di^b, one to Rhc and Fy^a antigens.

TABLE 1. Observed Genotype Frequencies in Blood Donors and Patients

Genotype	Donors (<i>N</i> = 400)		Patients (<i>N</i> = 79)	
	Frequency	<i>n</i>	Frequency	<i>n</i>
<i>Rh</i> system				
<i>RHD</i> *Ψ+	0.0100	4	0	79
<i>RHD</i> *Ψ−	0.9900	396	1	0
<i>RHD</i> *+	0.8625	345	0.8734	69
<i>RHD</i> *−	0.1375	55	0.1266	10
<i>RHCE</i> * <i>CC</i>	0.1750	70	0.1772	14
<i>RHCE</i> * <i>Cc</i>	0.4275	171	0.4051	32
<i>RHCE</i> * <i>cc</i>	0.3975	159	0.4177	33
<i>RHCE</i> * <i>EE</i>	0.0225	9	0.0253	2
<i>RHCE</i> * <i>Ee</i>	0.2575	103	0.3291	26
<i>RHCE</i> * <i>ee</i>	0.7200	288	0.6456	51
<i>Duffy</i> system				
<i>FY</i> *01/ <i>FY</i> *01	0.1260	50	0.1646	13
<i>FY</i> *01/ <i>FY</i> *02	0.4800	192	0.4177	33
<i>FY</i> *02/ <i>FY</i> *02	0.3950	158	0.4177	33
GATA-33T/T	0.7800	312	0.6962	55
GATA-33C/C	0.0250	10	0.0633	5
GATA-33T/C	0.1950	78	0.2405	19
<i>Kidd</i> system				
<i>JK</i> *01/ <i>JK</i> *01	0.2725	109	0.2785	22
<i>JK</i> *01/ <i>JK</i> *02	0.4800	192	0.5190	41
<i>JK</i> *02/ <i>JK</i> *02	0.2475	99	0.2025	16
<i>Kell</i> system				
<i>KEL</i> *01/ <i>KEL</i> *01	0.0025	1	0	0
<i>KEL</i> *01/ <i>KEL</i> *02	0.0500	20	0.0886	7
<i>KEL</i> *02/ <i>KEL</i> *02	0.9475	379	0.9114	72

N, number of individuals; *n*, number of alleles.

TABLE 2. Blood Group Alloantibodies Detected in 8 of the 79 Patients who Received Multiple Transfusions

Antibodies	Number of patients
Anti-D	1
Anti-E	1
Anti-Di ^a	1
Anti-Kp ^a	1
Anti-C, -E	2
Anti-E, -K, -Di ^b	1
Anti-c, -Fy ^a	1
Total	8

Correlation Between Phenotype and Genotype of the 38 Polytransfused Patients

Phenotype and genotype results from the transfused patients are shown in Table 3. We found discrepancies on Rh, Kell, Duffy, and Kidd systems in 16 of the 38 patients chronically transfused. The genotype of these patients was confirmed by another method, the Bead-Chip DNA analysis (Bioarray Solutions). These patients had been receiving transfusion more frequently than those who did not present discrepancies, and were the

TABLE 3. Phenotyping and Genotyping Discrepancies Found for 16 Patients

Genotype	Phenotype			
<i>Rh</i> system	RhD+	RhD−		
<i>RHD</i> *+	31			
Partial <i>RHD</i> *	2			
<i>RHD</i> *−		5		
	Rh C+c−	Rh C+c+	Rh C−c+	
<i>RHCE</i> * <i>CC</i>	3	2		
<i>RHCE</i> * <i>Cc</i>		18		
<i>RHCE</i> * <i>cc</i>			15	
	Rh E+e−	Rh E+e+	Rh E−e+	
<i>RHCE</i> * <i>EE</i>	2	1		
<i>RHCE</i> * <i>Ee</i>		10	1	
<i>RHCE</i> * <i>ee</i>		2	22	
<i>Kell</i> system	K+k−	K+k+	K−k+	
<i>KEL</i> *01/ <i>KEL</i> *01				
<i>KEL</i> *01/ <i>KEL</i> *02		3	1	
<i>KEL</i> *02/ <i>KEL</i> *02			34	
<i>Kidd</i> system	Jk(a+b−)	Jk(a+b+)	Jk(a−b+)	
<i>JK</i> *01/ <i>JK</i> *01	9			
<i>JK</i> *01/ <i>JK</i> *02	1	21		
<i>JK</i> *02/ <i>JK</i> *02		2	5	
<i>Duffy</i> system	Fy(a+b−)	Fy(a+b+)	Fy(a−b+)	Fy(a−b−)
<i>FY</i> *01/ <i>FY</i> *01 (T/T)	2	2		
<i>FY</i> *01/ <i>FY</i> *02 (T/T)		12		
<i>FY</i> *01/ <i>FY</i> *X (T/T)	1 ^a			
<i>FY</i> *01/ <i>FY</i> *02 (T/C)	2 ^a			
<i>FY</i> *02/ <i>FY</i> *02 (T/T)		2	5	
<i>FY</i> *02/ <i>FY</i> *02 (T/C)			7	
<i>FY</i> *02/ <i>FY</i> *02 (C/C)				4 ^a
<i>FY</i> *02/ <i>FY</i> *X (T/C)				1 ^a

^a*FY**B was not phenotypically expressed due to the GATA mutation and/or mutations in *FY* gene responsible for the Fy^{bw} phenotype (*FY**X).

only ones who had received transfusion within the previous 3 months.

Rh System

Presence or absence of *RHD*

Agreement between phenotype and genotype was observed for RhD on 36 samples. Thirty-one samples were both phenotyped and genotyped as RhD-positive (had amplified product from both *RHD* and *RHCE*) and five samples were phenotyped and genotyped as RhD-negative (had amplified product from *RHCE* but not from *RHD*). Two samples phenotyped as RhD-positive presented discrepancies between intron 4 and exon 7 analyses of *RHD*. One sample was further genotyped as partial D category V and another one as

partial D category DIIIc. The patient with partial D category V had anti-D in his serum.

RHCE*C/c

Thirty-six of the 38 samples agreed between phenotype and genotype. Two discrepant samples were phenotyped as RhCc and genotyped as *RHCE*CC*.

RHCE*E/e

Thirty-four samples had concordant phenotype and genotype. Two discrepant samples were phenotyped as RhE/e and genotyped as *RHCE*e/e*. One sample was phenotyped as RhEe and genotyped as *RHCE*EE* and one sample was phenotyped as Rhee and genotyped as *RHCE*Ee*.

Kell, Kidd, and Duffy Systems

Kell

There was an agreement between phenotype and genotype results for Kell on 37 samples. One discrepant sample was phenotyped as K-k+ and genotyped as *KEL*01/KEL*02*.

Kidd

In the Kidd system, phenotype/genotype did not correlate in three of the 38 samples. Two were phenotyped as Jk(a+b+) and were genotyped as *JK*02/JK*02* and one sample was phenotyped as Jk(a+b-) and genotyped as *JK*01/JK*02*.

Duffy

Twenty-six samples agreed between phenotype and genotype. Two discrepant samples were phenotyped as Fy(a+b+) and genotyped as *FY*01/FY*01*, and two samples were phenotyped as Fy(a+b+) and genotyped as *FY*02/FY*02*. The other eight discrepant samples occurred due to the presence of mutated GATA box and/or the SNP 265T responsible for the Fy^{bw} phenotype (*FY*X*) (Table 3). The correlation between Duffy phenotype and genotype confirmed previous observations showing that a substantial number of individuals with *FY*02* genotype do not express this antigen on the surface of their RBCs. Thus, appropriate correlation between genotype and phenotype required complete analysis of the *FY* polymorphisms 125G>A (*FY*01/FY*02*), 265C>T (associated with Fy^{bw} phenotype), and analysis of mutation in the GATA box (-33T>C). In this study, 14 of 34 samples with *FY*B* genotype had mutated GATA box, one sample Fy(a+b-) was *FY*A/FY*B* normal GATA, but had 265T (*FY*X*), and one

sample Fy(a-b-) was *FY*02/FY*02* heterozygous GATA mutation and heterozygous 265C/T.

DISCUSSION

This study demonstrates the relevance of performing molecular analysis for the determination of minor RBC antigens in transfusion-dependent patients such as patients with hematological diseases and renal failure. By employing PCR-RFLP assays, we have shown that there are mistyping when hemagglutination is performed to determine the blood group of patients who have been recently transfused with multiple units of donor RBCs (9,10).

As observed in genotype and phenotype results correlation of the 38 transfused studied patients, the discrepancies were found in 16 cases. Eight of the discrepancies occurred in the Rh system, one in the Kell system, three in the Kidd system, and four in the Duffy system. Indeed, the 16 discrepant results were confirmed by using a commercial Kit, the HEA BeadChip (Bioarray Solutions), that has been used for large scale of blood group DNA typing to increase the inventory of donors to the polytransfused patients and to facilitate the matching of RBC components to the recipient's blood type (20).

The relevance of genotype determination of minor RBCs for the management of multiply-transfused patients has been demonstrated by allowing the determination of the true blood group genotype, and by assisting in the identification of suspected alloantibodies and the selection of antigen-negative RBCs for transfusion.

Our data also show that PCR can be used to find RBC compatible units for patients by selecting regional blood donors based on the ABO/Rh phenotype because we did not find significant differences between the donors and patients genotype frequencies, although the patients were diverse and each one needed a different phenotype.

In a previous *FY* genotyping study in Brazilians, it was verified that 36% of the *FY*02* genes were not phenotypically expressed due to mutations in the *FY*02* allele. In Brazilian population, Fy(b+) blood has a prevalence of 65%, whereas Fy(b-) has a prevalence of 35%, as demonstrated by serological testing (25).

The genotyping for *FY* for 38 transfused patients showed that in eight of these patients, *FY*02* was not phenotypically expressed due to the GATA mutation and/or mutations in *FY* gene responsible for the Fy^{bw} phenotype (Table 3). This study again illustrates the relevance of molecular testing in this context.

Fy^{bw} is characterized by weak expression of *FY*02* that can only be detected using potent anti-Fy^b reagents. Unfortunately, reagents capable of detecting Fy^{bw} are

not available. Taking into consideration the expression of *FY*01* allele, two in five patients phenotyped as Fy(a+b-) require Fy(a+b-) blood component, and three of five could receive Fy(a+b+), Fy(a+b-) and Fy(a-b+). If we take in consideration the absence of Fy^b expression, eight of ten Fy(b-) could receive Fy(b+) blood component because of the presence of Fy^{bw} or Fy^b in nonerythroid cells (when GATA mutation is involved). As these transfusion-dependent patients are at risk of alloimmunization and most of them already have existing alloantibodies against RBC antigens. As they were transfused with antigen-matched blood units, the possibility to select Fy(b+) units can increase the availability of blood to them.

The contribution of genotyping to the management of polytransfused patients is also illustrated by the two patients' samples phenotyped as RhD-positive but genotyped as partial D. Patients with partial D are at risk for anti-D alloimmunization and would benefit from receiving D-negative RBCs for transfusion. Current D typing reagents in use in transfusion services may have difficulty in determining partial D individuals, so the genotyping leads to improved patient care and presumed less D alloimmunization.

As previously discussed, the seriousness of the alloimmunization problem has led to recommendations that hematological patients be transfused with blood of donors whose RBC antigens are more closely matched to those of the recipients (3-5). However, accurate antigen typing in transfused patients is a major problem due to the presence of donor RBCs in patient's circulation. On the basis of these and previous results (9,10,20) and under the test conditions we established, we recommend the addition of blood group genotyping for transfused patients to provide antigen-matched RBC transfusions.

The possibility to have an alternative to hemagglutination tests to determine the patient's antigen profile should be considered for patients who need repeated transfusion therapy. As automated procedures attain higher and faster throughput at lower cost, blood group genotyping is likely to become more widespread.

The implementation of automated platforms allows all donors to be genotyped, expanding the blood donor extended antigen database and the reduction of extended phenotype testing (23,24,26). With this additional tool, more donors presenting with rare and uncommon antigens are discovered, which improves the likelihood that patients requiring frequent transfusions will be provided with antigen-specific blood.

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REFERENCES

1. Perkins HA. The safety of the blood supply: Making decisions in transfusion medicine. In: Nance SJ, editor. *Blood Safety: Current Challenges*. Bethesda: American Association of Blood Banks, 1992. p 125-150.
2. Coles SM, Klein HG, Holland PV. Alloimmunization in two multitransfused patient populations. *Transfusion* 1981;21:462-466.
3. Michail-Merianou V, Pamphili-Panouspoulou L, Piperi-Lowes L, Pelegrinis E, Karaklis A. Alloimmunization to red cell antigens in thalassemia: Comparative study of usual versus better-match transfusion programmes. *Vox Sang* 1987;52:95-98.
4. Tahhan RH, Holbrook CT, Braddy LR, Brewer LD, Christie JD. Antigen-matched donor blood in the transfusion management of patients with sickle cell disease. *Transfusion* 1994;34:562-569.
5. Ambruso DR, Githens JH, Alcorn R, et al. Experience with donors matched for minor blood group antigens in patients with sickle cell anemia who are receiving chronic transfusion therapy. *Transfusion* 1987;27:94-98.
6. Avent ND. Large-scale blood group genotyping clinical implications. *Br J Haematol* 2009;144:3-13.
7. Pellegrino Jr J, Castilho L, Rios M, de Souza CA. Blood group genotyping in a population of highly diverse ancestry. *J Clin Lab Anal* 2001;15:8-13.
8. Legler TJ, Eber SW, Lakomek M, et al. Application of *RHD* and *RHCE* genotyping for correct blood group determination in chronically transfused patients. *Transfusion* 1999;39:852-855.
9. Castilho L, Rios M, Bianco C, et al. DNA-based typing for the management of multiply-transfused sickle cell disease patients. *Transfusion* 2002;42:232-238.
10. Castilho L, Rios M, Pellegrino Jr J, Saad STO, Costa FF. Blood group genotyping facilitates transfusion of beta-thalassemia patients. *J Clin Lab Anal* 2002;16:216-220.
11. Daniels G, Finning K, Martin P, Soothill P. Fetal blood group genotyping from DNA from maternal plasma: An important advance in the management and prevention of haemolytic disease of the fetus and newborn. *Vox Sang* 2004;87:225-232.
12. Daniels G, Finning K, Martin P, Summers J. Fetal blood group genotyping. Present and future. *Ann NY Acad Sci* 2006;1075:88-95.
13. Lee TH, Donegan E, Slichter S, Bush MP. Transient increase in circulating donor leucocytes after allogeneic transfusions in immunocompetent recipients compatible with donor cell proliferation. *Blood* 1995;85:1207-1214.
14. Adams PT, Davenport RD, Rcardon DA, Roth MS. Detection of circulating donor white blood cells in patients receiving multiple transfusions. *Blood* 1992;80:551-555.
15. Lee TH, Paglieroni T, Ohro H, Holland PV, Bush MP. Long-term multi-lineage chimerism of donor leucocytes in transfused trauma patients. *Blood* 1996;88:265a-265a.
16. 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.
17. 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;40:936-942.
18. Veldhuisen B, van der Schoot CE, de Haas M. Blood group genotyping: from patient to high-throughput donor screening. *Vox Sang* 2009;97:198-206.
19. Anstee DJ. Red cell genotyping and the future of pretransfusion testing. *Blood* 2009;114:248-256.

20. Ribeiro KR, Guarnieri MH, da Costa DC, Costa FF, Pellegrino Jr J, Castilho L. DNA array analysis for red blood cell antigens facilitates the transfusion support with antigen-matched blood in patients with sickle cell disease. *Vox Sang* 2009;97:147–152.
21. Singleton BK, Green CA, Avent ND, et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the Rh D-negative blood group phenotype. *Blood* 2000;95:12–18.
22. Maaskant-van Wijk PA, Faas BH, de Ruijter JA, et al. Genotyping of RHD by multiplex polymerase chain reaction analysis of six RHD-specific exons. *Transfusion* 1998;38:1015–1021.
23. Hashmi G, Shariff T, Seul M, et al. A flexible array format for large-scale, rapid blood group DNA typing. *Transfusion* 2005;45:680–688.
24. Hashmi G, Shariff T, Yi Z, et al. Determination of 24 minor red blood cell antigens for more than 2000 blood donors by high-throughput DNA analysis. *Transfusion* 2007;47:736–747.
25. Castilho L. The value of DNA analysis for antigens in the Duffy blood group system. *Transfusion* 2007;47:28S–31S.
26. Denomme GA, Van Oene M. High-throughput multiplex single-nucleotide polymorphism analysis for red cell and platelet antigen genotypes. *Transfusion* 2005;45:660–666.