Artigo Especial

Genotyping of Kell, Duffy, Kidd and RHD in patients with β Thalassemia

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Determination of Rh, Kell, Duffy and Kidd phenotypes in addition to ABO is used to prevent the alloimmunization to red blood cells (RBCs) antigens and as part of the antibody identification process in patients with β Thalassemia. However, phenotyping in these patients can be time consuming and difficult to interpret. In these situations, it would be valuable to have an alternative to hemagglutination tests to determine the patient's antigen profile. We used PCR-RFLP to genotype such patients.

DNA was prepared from 50 patients with β Thalassemia who had been phenotyped by routine hemagglutination, and tested for Kell, Kidd, Duffy/ GATA mutation by PCR-RFLP. RHD/non-D was analysed by PCR product size associated to RHD gene sequence in intron 4 and exon 10/3'UTR. The genotyping assays were performed without knowledge of phenotype results. For RHD/non-D, 47 were RhD+ and RHD+/RHCE+, and 3 were RhD- and RHD-/RHCE+. *For Kell, 48 kk were* K2K2 *and 2 Kk were* K1K2. *For Duffy, of 44 samples that had normal GATA box, 8 Fy(a+b-) were* FYA/FYA, *15 Fy(a+b+) were* FYB/FYB, *and 19 Fy(a+b+) were* FYA/FYB*; of the other 4 samples 3 were* FYA/FYB *and heterozygous GATA mutation, and 1 Fy(a-b-) was* FYB/FYB, *homozygous GATA mutation. Two samples phenotyped as Fy(a+b-) that had normal GATA , presented the 265T/298A mutations and two samples phenotyped as Fy(a-b+) were genotyped was* FYA/FYB.. *For Kidd , 15 Jk(a+b) were* JKA/JKA, *12 Jk(a-b+) were* JKB/JKB, *and 20 Jk(a+b+) were* JKA/JKB. *Three samples phenotyped as JK(a+b+) were genotyped as* JKB/JKB. *Genotype is more accurate than phenotype for determination of blood groups in polytransfused patients with βThalassemia. Genotyping in these patients can be helpful to select antigen-negative RBCs for transfusion.*
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Key-words: *Genotyping, blood groups, system*

Introduction

The incidence of alloimmunization to RBC antigens other than ABO and D is particularly high in patients with hemoglobinopathies (1), reaching up to 18% in patients with β Thalassemia who have received transfusion(s) (2-4). Alloimmunization may cause a variety of problems and dilemmas in long-term medical and transfusional management (5, 6). Most of these problems pertain to finding appropriate antigen-negative blood for transfusion to alloimmunized patients.

Programs to prevent alloimmunization to

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RBC antigens have been designed and implemented to provide antigen-matched RBC transfusions to patients with Thalassemia, particularly those who are alloimmunized and/ or in need of chronic transfusion support (7, 8). In addition to ABO, RBCs are phenotyped for Rh, Kell, Duffy and Kidd prior to transfusion aiming both to prevent the alloimmunization to these RBC antigens, and as part of the antibody identification process (9).

Cell typing is essential in sensitized patients in order to confirm the identity of suspected alloantibodies, and to facilitate the identification of antibodies that may be formed in the future. However, accurate phenotyping of RBCs from transfused patients with Thalassemia is a very complex process due to the presence of transfused donors RBCs in the circulation of the recipient. Thus, in these patients phenotyping can be time consuming and difficult to interpret. It is also complicated to type cells when a patient's RBCs have a positive direct antiglobulin test and no direct agglutinating antibody is available.

The DNA technology led to the understanding of the molecular basis of many blood group antigens. The majority of blood group polymorphisms are associated with a single point mutation in the gene encoding the protein carrying the blood group antigen (10, 11). This knowledge allows the use of DNA testing to predict the blood group antigen profile of na individual, and that can be used to overcome the limitations of hemagglutination assays.

Several assays for blood group genotyping of patients have recently been developed with the goal of reducing risk or helping in the assessment of the risk of hemolytic disease of the newborn. However, these assays have not been generally applied to the genotyping of patients who have received recent or chronic transfusion care.

The dilema of using DNA obtained from a transfused patients' white blood cells (WBC) is that the donor leucocytes contained in the transfused units could, at least theoretically, interfere with genotyping results (12, 13, 14). However, studies performed with patient's WBC samples have shown that these cells can be used reliably to determine a blood group polymorphism by polymerase chain reaction (PCR)-based assays even when blood samples from recently transfused patients are used as the source of DNA (15, 16). This study demonstrates that post-transfusion blood samples can be safely used for genotyping blood groups. That eliminates the need to use current time-consuming and frequently ineffective methods that involve separation of patient reticulocytes from donor mature RBCs.

We performed genotype assays and correlated the results with phenotype results for the blood groups *RH D*, *KEL*, *JK* and *FY* using WBCs from polytransfused patients with β Thalassemia. We also report here that WBCs can be reliably employed as source of DNA for PCR-based assays for blood group genotyping in transfused patients with β Thalassemia as compared against PCR results obtained from other cell source of DNA. Moreover, we observed that genotype is more accurate than phenotype for determination of blood groups in polytransfused patients with β Thalassemia.

Methods

Samples: Blood samples and buccal epithelial cells were collected from each patient after informed consent was given to the health care physician or specialized nurse.

Blood samples: We studied samples from 50 patients with β Thalassemia who have been given transfusions of antigen-matched RBC units at the Hemocentro (Unicamp, Campinas, Brazil). These patients had been transfused at least 3 occasions and their blood samples were phenotyped at the time of each transfusion. The most recent blood sample from each patient was also genotyped for *RHD*, *KEL*, *JK* and *FY*. Blood samples from 100 normal blood donors who had been previously phenotyped for Rh, Kell, Kidd and Duffy were tested as controls.

Buccal epithelial cells: Buccal epithelial cells samples were tested for the patients whom had phenotype/genotype discrepancies. We obtained buccal epithelial cells by wiping the mouth mucosa with a cotton wool swab.

Agglutination tests: At all times phenotypes were determined by hemagglutination in gel cards (Diamed AG, Morat, Switzerland) using two anti-sera sources.

DNA preparation: DNA was extracted from blood samples using either the phenol-chloroform method (17) or the Easy DNA Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. DNA from buccal epithelial cells contained in the cotton wool swabs was extracted by the Easy DNA Kit according to manufacturer's protocol #3.

Polymerase chain reaction (PCR) amplification: The primers and amplification conditions used, were the same as previously published (15, 16). Briefly, the PCR was performed with 100-200 ng of DNA, 50 pmole of each primer, 2 nmole of each dNTP, 1.0 U *Taq* DNA polymerase and buffer in a final volume of 50 µl. The PCR analysis for the presence of *RHD* was performed in two genomic regions, intron 4 and exon 10, as previously described (16). For intron 4, three primers (RHI41, RHI42 and RHI43) yielded a product of 115 bp for *RHD* and 236 bp for *RHCE*. For exon 10, a common 5'primer (EX10F) was used for both *RHD* and *RHCE*. When paired with the RHD-specific 3'-untranslated region (UT) primer (RHD3UT), it produced a product of 245 bp, and when paired with the RHCE-specific 3'-UTR (RHCE3UT), it yielded a product of 160 bp (Figure 1).

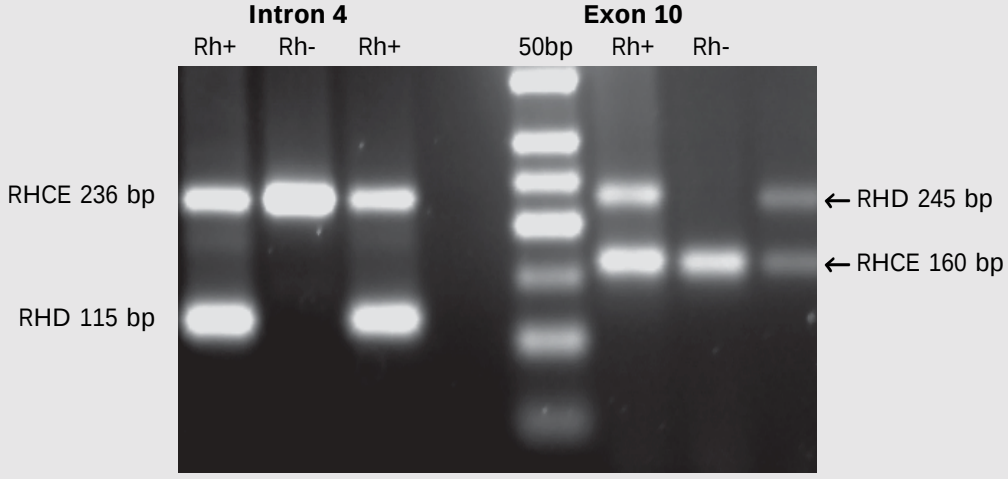

Figure 1. *RH D genotyping*

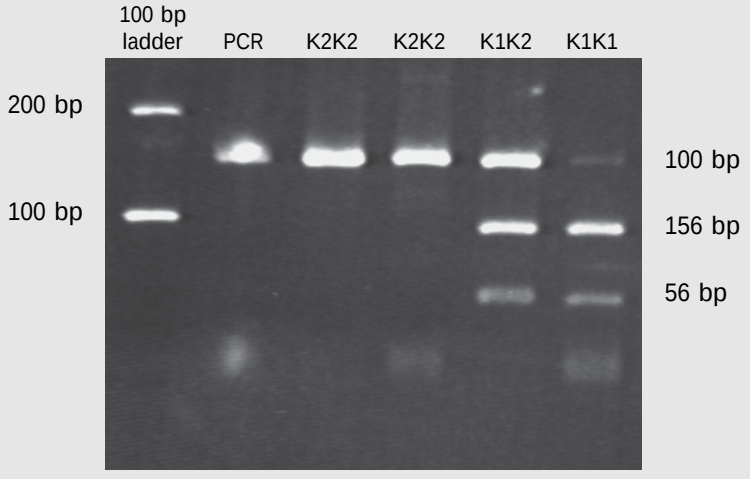

Figure 2. *Kell genotyping*

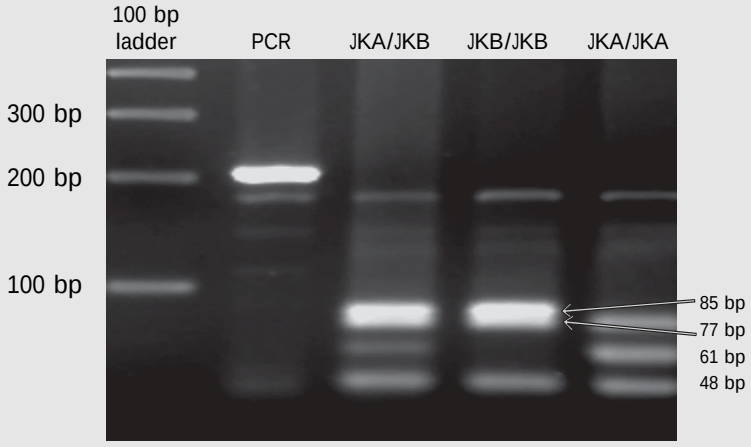

Figure 3. *Kidd genotyping*

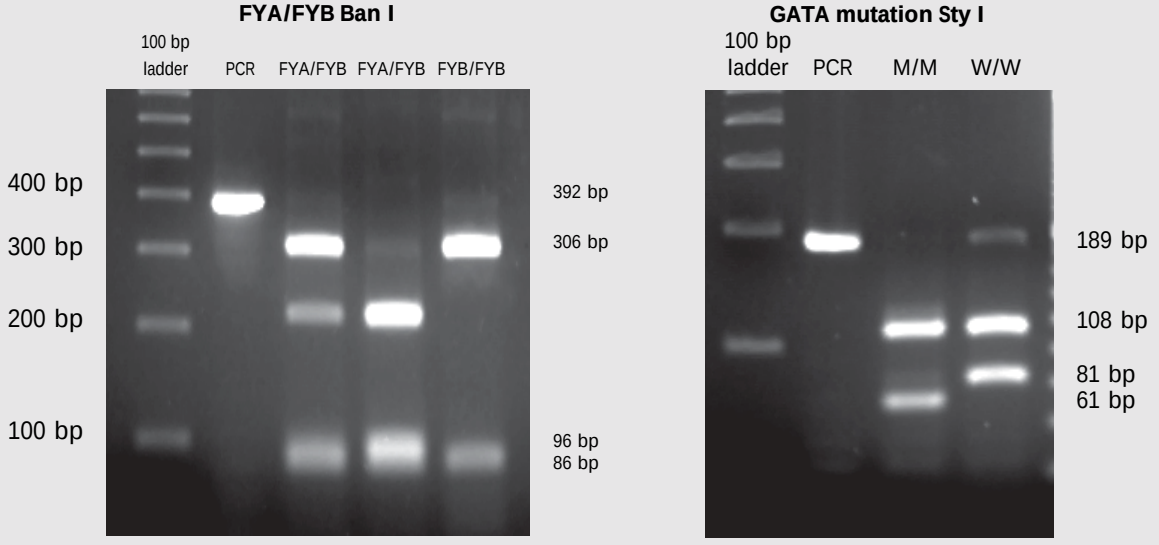

Figure 4. *Duffy genotyping*

RFLP analysis: The 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 20ml using 10ml of amplified product and enzyme in 1x buffer according to manufacturer's instructions.

The enzymes *Bsm* I, *Mnl* I and *Ban* I were used to determine, respectively *KEL 1/ KEL 2* (698C>T), *JK A/JK B* (838^{A}>G) and *FY A/ FY B* (125 G>A) (18-22). (Figures 2, 3, 4) Furthermore, the *Sty* I enzyme was used to distinguish between normal and mutated GATA-1 binding motif (-33T>C), because the GATA-1 binding site is critical for expression of Fy^{b} protein in the red cell membrane (23) (Figure 4).

Results

Correlation between phenotype and genotype of the 50 patients with β Thalassemia were as follows for each blood antigen studied: Phenotype and genotype results from the patients with β Thalassemia are shown in table 1. Among the 50 patients, 5 had phenotype/genotype discrepancies.

Presence or absence of *RHD*: 47 of the 50 samples were both phenotyped and genotyped as RhD-positive (had amplified product from both *RHD* and *RHCE)* and 3 samples phenotyped and genotyped as RhD-negative (had amplified product from *RHCE* but not from *RHD*).

Kell: we observed 100% correlation between phenotype and genotype results for Kell: 48 samples phenotyped as k/k were genotyped as *K2/K2* and two samples phenotyped as K/k were genotyped as *K1/K2*.

Kidd: phenotype/ genotype did not correlate in 3 of the 50 samples, as they phenotyped as Jk(a+b+) and genotyped as *JKB/JKB*.

Duffy: 44 samples had normal GATA box, 8 Fy(a+b–) were *FYA/FYA*, 15 Fy(a–b+) were *FYB/FYB*, 19 Fy(a+b+) were *FYA/FYB* and two samples phenotyped as Fy(a+b) were Fy^x and, presented the 265T and 298A mutations. Of the other 4 samples 3 Fy(a+b–) were *FYA/FYB* and heterozygous GATA mutation, 1 Fy(a–b–) was *FYB/FYB* and homozygous GATA mutated. The two discrepant samples phenotyped as Fy(ab+) and were genotyped as *FYA/FYB*, homozygous GATA mutation.

Correlation between phenotype and genotype of the 100 volunteer blood donors control:

In this group was observed an agreement of 100% for all blood groups studied.

Genotypes results obtained on DNA from buccal cells and WBCs from the 6 patients whom had phenotype/genotype discrepancies:

In order to demonstrate the absence of microchimerism in discrepant results, all 5 patients with phenotype/genotype discrepancies results had the DNA from buccal cells tested by PCR-RFLP, and the results were identical to those obtained when tests were performed using DNA from blood samples.

Discussion

This study shows the relevance of performing molecular analysis for the determination of blood groups in transfusion dependent patients such as patients with β Thalassemia. By employing PCR-RFLP assays we have shown that as expected there is mistyping when hemagglutination is performed to determine the blood group of multitransfused patients. As observed in genotype and phenotype results correlation of the 50 transfused patients studied, phenotype/genotype discrepancies were found in 5 cases.

We confirmed previous study observations that DNA prepared from blood samples collected from a patient who has recently

Table 1: *Genotyping results for, RH, KEL, KIDD and DUFFY in samples from 50 patients with β Thalassemia*

Genotype	**Phenotype**			
Rh System	**RhD+**	**RhD-**		
RHD+/RHCE+	47	0		
RHD–/RHCE+	0	3		
Kell System	**K+k+**	**K-k+**		
K1K2	2	0		
K2K2	0	48		
Kidd System	**Jk(a+b–)**	**Jk(a+b+)**	**Jk(a–b+)**	
JKA/JKA	15	0	0	
JKA/JKB	0	20	0	
JKB/JKB	0	3	12	
Duffy System	**Fy(a+b-)**	**Fy(a+b+)**	**Fy(a-b+)**	**Fy(a-b-)**
FYA/FYA (T/T)	8	0	0	0
FYA/FYB (T/T)	0	19	2	0
FYA/FYB (T/C)	3	0	0	0
FYB/FYB (T/T)	2*	0	15	0
FYB/FYB (C/C)	0	0	0	1

* Fy^x *265T, 298*^{A}

received a transfusion, can be used for blood group genotyping without risk of detecting microchimerism (16).

The relevance of performing molecular tests in transfusion dependent patients is well illustrated in the Duffy system. In the presence of nomal GATA-1 binding motif, phenotypes and genotypes agree, but when the GATA-1 motif is mutated a pseudo-discrepancy is observed. That is due to the absence of the FY gene expression in the erythroid lineage. However, patients carrying such phenotypes do not need blood units of negative phenotype, once that this protein is expressed in other body tissues, thus recognized as self. This small change in blood type requirement increases the availability of special blood types for such patiens. GATA mutation in our population has only been associated with the FY B allele, however, it has been found associated with the FY A allele in population from Papua New Guinea (24). Our study also identified two true discrepant samples. Their hemagglutination results were Fy(a–b+) and their genotype results were *FY A/FY B*. Accordingly, DNA samples obtained from buccal epithelial cells confirmed the genotype obtained from the blood sample. The phenotype performed in segments of the transfused units further confirmed that the Fy(ab+) reflected the donor's blood type. Three samples also presented discrepancy in the Kidd typing. All 3 samples phenotyped as Jk(a+b+) were genotyped as *JKB/JKB.* The absence of *JK A* was confirmed by genotyping DNA samples obtained from buccal epithelial cells, and by performing phenotyping in segments of the transfused units, that the Jk(a+) reactivity was due to donors RBCs in the patient's circulation system. These three patients could have been immunized for Jk^a antigen because they were receiving antigen-matched RBC units based in the phenotyping interpretation.

As previously discussed, the seriousness of the alloimmunization problem has led to recommendations that patients with β Thalassemia be transfused with blood of donors whose RBC antigens are more closely matched to those of the recipients (7-9). However, accurate antigen typing in transfused patients is a major problem due to the presence of donor RBCs in patient's circulation. Based on our results and under the test conditions we established, we recommend the addition of blood group genotyping for transfused patients to provide antigen-matched RBC transfusions. However, caution in performing these tests and interpreting results must be exercized, since the PCR-based technique is prone to contamination, and the presence of a particular genotype may not be associated with that antigen expression on the RBC's membrane. Such situations include, the detection of genes with a silencing mutation in a location other than that being analysed (e.g., point mutation in the GATA box), the detection of a gene that is silenced by na alteration of a gene encoding protein with a modifying effect (e.g. Rhmod, Rhnull), or the detection or nondetection of a hybrid gene (25-29). The possibility to have na alternative to hemmaglutination tests to determine the patient's antigen profile should be considered for patients with β Thalassemia who need repeated transfusion therapy.

Genotipagem dos sistemas Kell, Duffy, Kidd e RHD em pacientes com β Talassemia

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Sumário

A determinação dos fenótipos Rh, Kell, Duffy e Kidd, associada ao ABO é utilizada para prevenir a aloimunização a antígenos eritrocitários e participam também no processo de identificação de anticorpos nos pacientes com β talassemia.

Todavia, a fenotipagem desses pacientes é trabalhosa e de difícil interpretação. Nesta situação, deve ser avaliada uma alternativa ao teste de hemaglutinação para determinar o padrão antigênico dos pacientes.

Utilizamos para tal fim o método PCR-RFLP. Foram preparados DNAs de 50 pacientes com β talassemia que haviam sido anteriormente fenotipados pela hamglutinação e testados para Kell, Kidd, Duffy/GATA mutação por PCR-RFLP. RHD/não-D foi analisado pelo tamanho do produto, do PCR associado à seqüência do gene RHD no intron 4 e exon 10/3' UTR.

Os testes de genotipagem foram realizados sem o conhecimento dos resultados dos fenótipos. Para os RHD/não-D, 47 foram RhD+ e RHD+/RHCE+, e 3 foram RhD- e RHD-/RHCE+.
Para o Kell, 48 kk foram K2K2 e 2 Kk foram K1K2.
Para o Duffy, das 44 amostras que haviam sido normais, GATA box, 8 Fy(a+b-) foram FYA/FYA, 15 Fy(a+b-) foram FYB/FYB e 19 Fy(a+b+) foram FYA/FYB; das outras 4 amostras, 3 foram FYA/FYB e heterozigoto GATA mutação, e 1 Fy(a-b-) era FYB/FYB, homozigoto GATA mutação.
Duas amostras fenotipadas como Fy(a+b-), que eram normais GATA, apresentavam as mutações 265T/298^{A} e 2 amostras fenotipadas como Fy(a-b+) haviam sido genotipadas como FYA/FYB.
Para o Kidd, 15 Jk(a+b-) foram JKA/JKA, 12 Jk(a-b+) foram JKB/JKB, e 20 Jk(a+b+) foram JKA/JKB. Três amostras fenotipadas como JK(a+b+) haviam sido genotipadas como JKB/JKB.
A genotipagem é mais acurada que a fenotipagem para determinação de grupos sangüíneos em pacientes portadores de β talassemia politransfundidos. A genotipagem nesses pacientes pode ser importante para selecionar hemácias antigenicamente negativas para transfusão de glóbulos vermelhos.
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Palavras-chave: *Genotipagem, grupos sanguínios, sistema*

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