DNA-based typing of blood groups for the management of multiply-transfused sickle cell disease patients

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BACKGROUND: The usefulness of DNA genotyping for RBC antigens as a tool for the management of multiply-transfused patients with sickle cell disease (SCD) to overcome the limitations of hemagglutination assays was evaluated.

STUDY DESIGN AND METHODS: Blood samples from 40 multiply-transfused SCD patients were studied by hemagglutination and by PCR-RFLP for antigens or genes in the Rh (D, C/c, E/e), Kell, Kidd, and Duffy systems.

RESULTS: Discrepancies were found between hemagglutination and DNA typing test results in six patients: two were discrepant in Rh typing (one was D− by hemagglutination and *RhD* by DNA, and one was E+e− and *RhEe* by DNA), two were discrepant in Duffy typing [both were Fy(a+b−) and Fy^b/Fy^b by DNA], and four were discrepant in Kidd typing [Jk(a+b+) and Jk^b/Jk^b by DNA; two of these samples were also discrepant in Duffy]. Stored segments from blood units that had been recently transfused to these six recipients were phenotyped, confirming that the transfused RBCs were the source of the discrepancy between genotype and phenotype.

CONCLUSION: DNA typing of blood groups by PCR-RFLP in peripheral blood WBCs contributes to the management of transfusions in SCD patients by allowing a more accurate selection of donor units.

Sickle cell disease (SCD) is a common hemoglobinopathy that affects approximately 0.2 to 0.5 percent of blacks.[1] RBC transfusion is a key part of the management of some SCD patients.[2,3] The major risks of transfusions are unexpected incompatibility reactions[4] and the transmission of infectious agents. Iron overload and alloimmunization are also frequently observed among some categories of chronically transfused patients. Alloimmunization leads to an increased risk of transfusion reactions, reducing the available pool of compatible blood for transfusion in subsequent crises. The incidence of alloimmunization to RBC antigens other than A, B, and D is particularly high among patients with hemoglobinopathies,[5] reaching 36 percent in SCD patients who have received previous transfusions.[6-12] This high rate is mainly caused by differences in the frequencies of RBC antigens between blood donors of European descent and SCD patients of African descent.[11,13-15] Alloimmunization is the source of a variety of problems during long-term medical and transfusion management, with the main problem being the identification of appropriate antigen-negative RBCs for transfusion.[16]

Determining the blood group of SCD patients by hemagglutination has been the classic method for identification of suspected alloantibodies and for prediction

ABBREVIATION: SCD = sickle cell disease.

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of the specificity of antibodies likely to develop in the future. However, accurate phenotyping of SCD patients is often complicated either by the presence of transfused donor RBCs in the recipient's circulation, by positive DATs, or by the lack of available direct agglutinating antibodies. DNA technology has led to the understanding of the molecular basis of almost all clinically relevant blood group antigens, making it possible to overcome the limitations of hemagglutination assays.[17,18]

Several assays for blood group genotyping have recently been developed and are being used for the assessment of the risk of HDN.[19-22] The use of peripheral blood WBCs as a source of DNA for the genotyping of multiply-transfused patients has generated some concern because of the theoretical risk of contamination of the patient specimen with donor WBCs.[23-26] Recent reports show that blood samples from transfused patients can be safely used for DNA typing of blood groups because the amount of patient's DNA far exceeds that in the donor WBCs,[21,22] eliminating the need for time-consuming and frequently ineffective methods of separation of patient's reticulocytes from mature RBCs.

This study evaluated the contribution of DNA genotyping for RBC antigens as a tool for the management of multiply-transfused SCD patients in order to overcome the limitations of hemagglutination assays. We studied blood samples from 40 multiply-transfused SCD patients by hemagglutination and by PCR-RFLP for antigens in the Rh (D, C/c, E/e), Kell, Kidd, and Duffy systems and found discrepancies between hemagglutination and DNA typing test results in samples from six patients. Our observation confirmed results from several previous studies that peripheral blood WBCs from multiply-transfused SCD patients could be reliably employed as a source of DNA for the PCR-based assays. Furthermore, we observed that taking genotype into account allowed better selection of compatible units for patients with discrepancies between genotype and phenotype, leading to increased cell survival and a reduction of the transfusion frequency.

MATERIALS AND METHODS

Patients

We studied peripheral blood samples from 40 SCD patients (HbSS) who received transfusions of RBC units matched for antigens in the Rh, Kell, Duffy, and Kidd blood group systems at Hemocentro (Unicamp, Campinas, Brazil) and who agreed to participate in this study by signing an institutional review board-approved informed consent. These patients were chronically transfused, and their blood samples had been phenotyped by hemagglutination at the time of their first transfusion at Hemocentro. All of the patients with discrepancies between genotype and phenotype had received at least three transfusions within the previous 3 months.

Three patients had alloantibodies in their serum (one anti-D and two anti-Jk^a). Two of them became alloimmunized to the detected phenotype [Jk(a+)]. The most recent blood sample from each of the alloimmunized patients was genotyped for *RhD, RhC/C, RhE/E, K, Jk,* and *Fy*.

Control group

We tested genotyped blood samples from 100 normal blood donors of African ethnicity who had been previously phenotyped for antigens in the Rh, Kell, Kidd, and Duffy systems as controls for our procedures. This control group was representative of the ethnic background of the patients.

Buccal epithelial cells

We collected buccal epithelial cells from all six patients for whom hemagglutination and DNA typing of peripheral blood generated discrepant test results by swiping the mouth mucosa with a cotton wool swab. DNA was extracted, and DNA typing was carried out as described later here.

Agglutination tests

Phenotypes were determined by hemagglutination in gel cards (Diamed AG, Morat, Switzerland) using two different commercial sources of antisera (Gamma Biologicals, Houston, TX, and Diamed AG).

DNA preparation

DNA was extracted from blood samples by using either the phenol-chloroform method[27] or a kit (Easy DNA kit, Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The DNA from buccal epithelial cells in the cotton wool swabs was also extracted with the kit (Easy DNA kit, Invitrogen) as previously reported.[19]

PCR amplification

The primers and the amplification conditions used for *RhE/E, K, Jk,* and *Fy* genotyping have been previously published.[19,21] Briefly, PCR was performed with 100 to 200 ng of DNA, 50 pmol of each primer, 2 nmol of each dNTP, 1.0 U *Taq* DNA polymerase, and buffer in a final volume of 50 μL. PCR was carried out in a thermal cycler (model 480, Perkin Elmer, Foster City, CA), and the same profile was used for all assays as follows: 15 minutes at 95°C; 35 cycles of 40 seconds at 94°C, 40 seconds at 62°C, and 1 minute at 72°C; followed by 10 minutes at 72°C.

Amplified products were analyzed by electrophoresis in 1.5-percent agarose gel in Tris-acetate EDTA buffer.

Allele-specific PCR

PCR analysis for the presence of *RhD* was performed in two genomic regions: intron 4 and exon 10. Exon 10 analyses were performed as previously reported.[24] A set of three primers, comprising RHI41 and RHI42 (previously reported),[21] and a third primer, RHI43 (5′-ATTAGCTGGGCATGGTGGTG-3′), were used for intron 4. The combination of these three primers generates products of 115 bp for *RhD* and 236 bp for *RhCE* and allows amplification of partially degraded DNA specimens such as those found in clinical settings and in buccal epithelial cell specimens with a low concentration of DNA (Fig. 1).

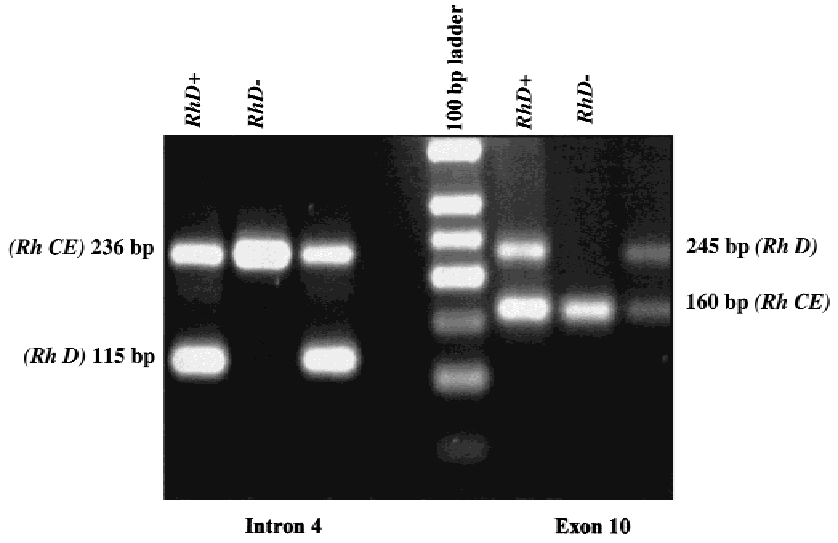

Fig. 1. PCR analyses for the presence of *RhD* in intron 4 and exon 10 sequences.

RFLP analysis

PCR-amplified products were digested overnight with the appropriate restriction enzymes[21] (MBI Fermentas, Amherst, NY, and New England Biolabs, Beverly, MA) in a final volume of 20 μL using 10 μL of amplified product, according to the enzyme manufacturers' instructions. The RFLP analyses were performed after electrophoresis in 3-percent agarose in Tris-acetate EDTA buffer or 8-percent PAGE in Tris-Borate-EDTA (Figs. 2 and 3).

Multiplex PCR

Analysis for *RhD* variants was performed in all samples by using a two-*RhD* multiplex assay. One, which is directed at six regions of *RhD,* covers all exons with *RhD*-specific sequences in the coding regions,[28] and the other, which detects the presence of D, differentiates *RhC/c* and identifies *RhDψ*[29] (Fig. 3).

Sequence analysis

Sequence analysis was performed on PCR products amplified from genomic DNA by using *RhD*-specific primers for exons 3, 4, 5 and 7, as previously re-

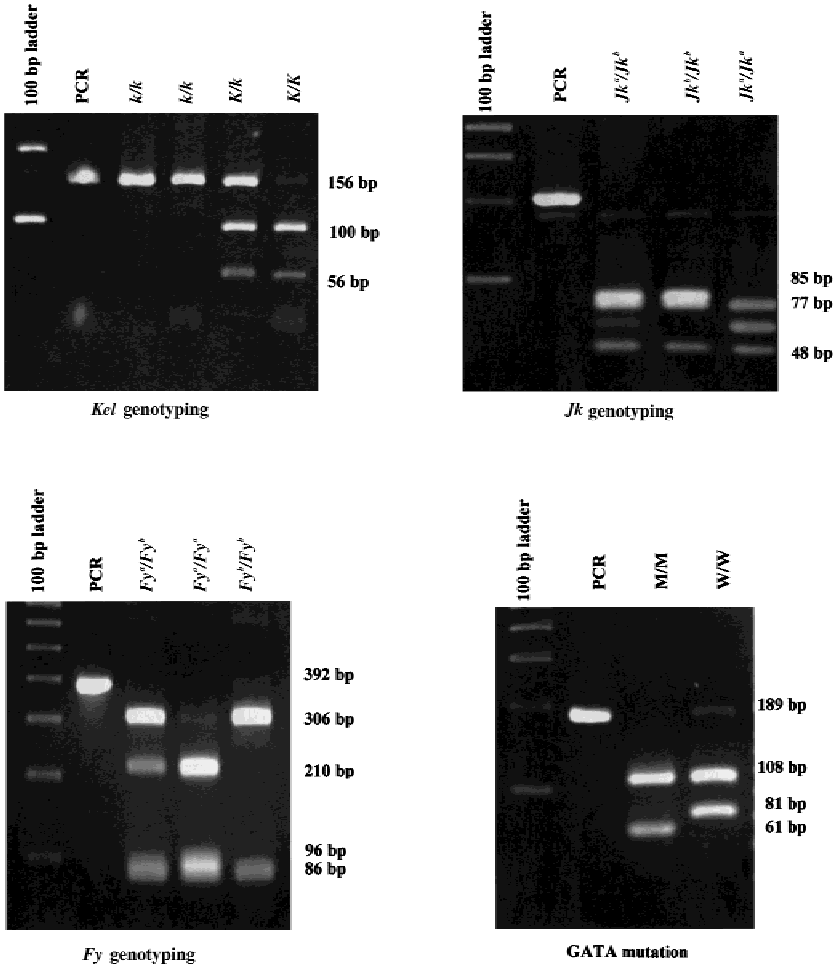

Fig. 2. RFLP analyses for *K, Jk,* and *Fy* genotyping. M/M = mutated, W/W = wild.

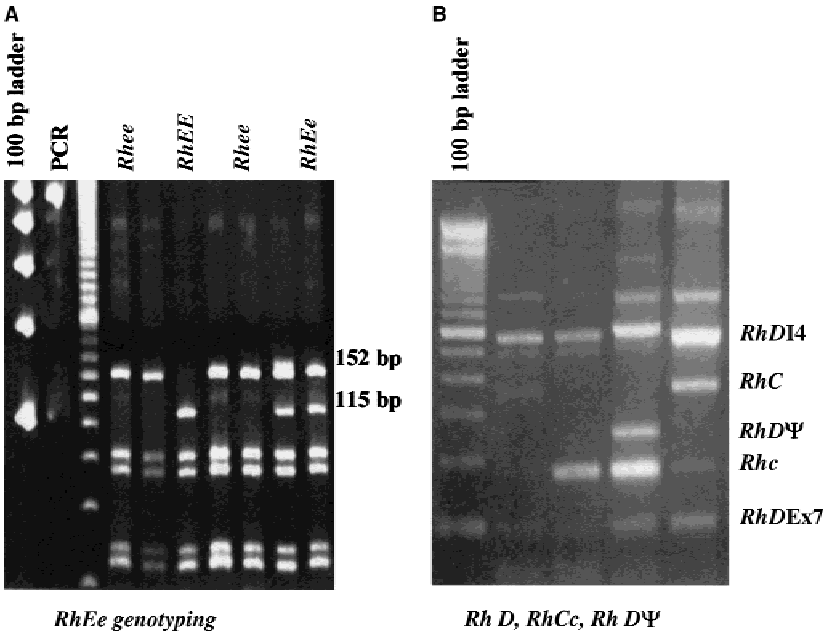

Fig. 3. (A) RFLP analysis for *RhEe* genotyping. (B) Multiplex PCR for predicting D and C and/or c phenotypes and for detecting the presence of *RhD*ψ.

TABLE 1. Phenotyping and genotyping results for antigens or genes in the Rh system on samples from 40 polytransfused SCD patients

Genotype	Phenotype		
Rh system	D+	D−	
RhD+/RhCE+	37	1	
RhD−/RhCE+	0	2	
	EE	Ee	ee
RhE/RhE	2	0	0
RhE/Rhe	1	5	0
Rhe/Rhe	0	0	32
	CC	Cc	cc
RhC/RhC	2	0	0
RhC/Rhc	0	11	0
Rhc/Rhc	0	0	27

ported.[30,31] For *RhD*-specific exon 3, we designed the reverse primer (RHDI3R: 5′-ATGTTGCCCAGCTCGGTCC-3′) specific for *RhD*-based sequences from GenBank (AB035186 and AB035187), taking advantage of the differences between *RhCE* and *RhD* in intron 3. This primer was used with the common (*RhD* and *RhCE*) sense primer (RHDCEX3F: 5′-TATTCGGCTGGCCATGA-3′).

PCR products were purified by elution from 1-percent agarose gels by using a gel extraction kit (Qiaex II, Qiagen, Valencia, CA) and were sequenced directly, without subcloning, on a sequencer (ABI 373XL, with Big Dye reagent BD Half-term, GenPak, Perkin Elmer Biosystems).

RESULTS

When genotype and phenotype results for *RhD, RhC/c, RhE/e, K, Jk, and Fy* were correlated among multiply-transfused SCD patients, six of the 40 SCD patients had discrepancies between phenotype and genotype (Tables 1 and 2). Figures 1 to 3 summarize the interpretation of the genotyping results. In contrast to the results for the SCD patients, there was complete agreement between phenotype and genotype in the control group of 100 normal blood donors of African ethnicity.

Rh system

A correlation between phenotype and genotype results showed concordance for C/c in 40 of 40 patients and for E/e in 39 of 40 patients; one discrepant sample was phenotyped as E+e− and was genotyped as *RhEe*.

Thirty-nine of the 40 samples had concordant phenotype and genotype results for *RhD:* 37 were D+, and two were D- in all assays. The discrepant sample phenotyped as D- and genotyped as *RhD*+. Genomic DNA analysis performed by sequence revealed the presence of 455A>C as heterozygous (specific for D^{IIIa}),[30] 602C>G and 667T>G as homozygous (common for both D^{IIIa} and DAR),[30,31] and 1025T>C as heterozygous (specific for DAR).[31] The same results were observed in PCR-RFLP for nt positions 667G/G (using *Hin*cII) and for 1025T/G (using *Hph*I).

Kell, Kidd, and Duffy systems

There was complete agreement between genotyping and serologic typing for K. In the Kidd system, four of the 40 SCD patients had samples phenotyped as Jk(a+b+) and genotyped as Jk^b/Jk^b. In the Duffy system, two samples were phenotyped as Fy(a+b−) and were genotyped as Fy^b/Fy^b, homozygous for the GATA mutation. These two samples also had discrepancies in the Kidd system typing and had anti-Jk^a in their serum, but not anti-Fy^a.

Genotype results obtained from DNA of buccal cells and peripheral blood from the six patients with phenotype and genotype discrepancies

To demonstrate the absence of microchimerism in patients with discrepant phenotype or genotype results, all six patients with discrepant results had DNA from buccal cells tested by PCR-RFLP. The results were identical to those obtained when tests were performed on DNA from peripheral blood samples of the same patient.

TABLE 2. Phenotyping and genotyping results for K/K, Jk^a/Jk^b, and Fy^a/Fy^b-GATA on samples from 40 polytransfused SCD patients

Genotype	Phenotype			
Kell system	K+k+	K–k+		
Kk	1	0		
KK	0	39		
Kidd system	Jk(a+b−)	Jk(a+b+)	Jk(a−b+)	
Jk^a/Jk^a	14	0	0	
Jk^a/Jk^b	0	15	0	
Jk^b/Jk^b	0	4	7	
Duffy system	Fy(a+b−)	Fy(a+b+)	Fy(a−b+)	Fy(a−b−)
Fy^a/Fy^a (T/T)*	1	0	0	0
Fy^a/Fy^b (T/T)*	0	8	0	0
Fy^a/Fy^b (T/C)†	10	0	0	0
Fy^b/Fy^b (T/T)*	0	0	4	0
Fy^b/Fy^b (C/C)‡	2	0	0	15

* T/T: −33 = normal GATA.
† T/C: −33T/C = heterozygous GATA mutation.
‡ C/C: −33 T/C = homozygous GATA mutation.

Control group

Complete agreement between phenotype and genotype was observed in the control group. The blood type distribution was the following:

Rh system. When tested for *RhD,* 93 of the 100 samples were *RhD+,* and 7 were *RhD−* in all assays. When tested for *RhEe,* 84 were *Rhee,* 12 were *RhEe,* and 4 were *RhEE*. When tested for *RhCc,* 62 were *Rhcc,* 31 were *RhCc,* and 7 were *Rhcc*.

Kell, Kidd, and Duffy systems. In the Kell system, 96 samples were *k/k* and 4 were *K/k*. In the Kidd system, 31 samples were Jk^a/Jk^a, 43 were Jk^a/Jk^b, and 26 were Jk^b/Jk^b. In the Duffy system, the 24 samples that phenotyped as Fy(a+b−) were Fy^a/Fy^b −33 T/C (heterozygous GATA mutation), the 24 that phenotyped as Fy(a+b+) were Fy^a/Fy^b −33 T/T (normal GATA), the 12 that phenotyped as Fy(a-b+) were Fy^b/Fy^b −33 T/T (normal GATA), and the 40 that phenotyped as Fy(a−b−) were Fy^b/Fy^b −33C/C (homozygous GATA mutation).

DISCUSSION

The serious consequences of alloimmunization have led some SCD treatment centers to select blood of donors with closely matched RBC antigens for transfusion into sickle cell recipients.[12-16] The success of this task depends on the ability to type multiply-transfused patients accurately. Molecular testing indicated that mistyping by hemagglutination occurred in 6 of our 40 multiply-transfused SCD patients. Three of these six patients had been transfused with phenotypically matched RBCs and developed alloantibodies (not autoantibodies) to the detectable phenotype. They had been receiving transfusion more frequently than those who did not present discrepancies and were the only ones who had received transfusion within the previous 3 months. Our data also confirm previous reports indicating that microchimerism does not affect blood group genotyping results obtained from peripheral blood samples of multiply-transfused patients, probably because of the overwhelming excess of patient DNA.[21,22] Indeed, none of the six discrepant results were due to DNA microchimerism, as confirmed by testing DNA from buccal epithelial cells.

In addition to its contribution to the general accuracy of identification of RBC antigens, genotyping of transfusion-dependent SCD patients allows assessment of the risk of alloimmunization against antigens in the Duffy system due to regulation of antigen expression determined by the GATA-1 box. In the presence of the normal GATA-1 binding motif, phenotype and genotype agree, but when the GATA-1 motif is mutated, a pseudo-discrepancy is observed because of the absence of *Fy* gene expression in the erythroid lineage.[32,33] Thus, patients phenotyped as Fy(b−) who carry the mutated GATA box can receive Fy(b+) blood units with a minimum risk of alloimmunization because Duffy protein is expressed in other body tissues.[32,33] Table 2 shows that 27 of the 28 patients phenotyped as Fy(b−) could receive Fy(b+) RBCs. Two discrepant specimens were phenotyped as Fy(a+b−) and were genotyped as homozygous for Fy^b. The genotype was confirmed in DNA samples obtained from buccal epithelial cells. Stored segments from blood units that had been recently transfused into these recipients were phenotyped, confirming that the transfused RBCs were Fy(a+b−) and were the source of the discrepancy. The GATA mutations observed in the studied population were associated with the Fy^b allele. However, it should be noted that GATA mutations have also been associated with the Fy^a allele in a population from Papua New Guinea.[34] Interestingly, these patients were not previously sensitized to Fy^a and had a discrepancy in Kidd system typing [they were Jk^b/Jk^b phenotyped as Jk (a+b+)]. Unfortunately, they were transfused with Jk(a+) units, and they developed anti-Jk^a.

A total of four samples phenotyped as Jk(a+b+) were genotyped as Jk^b/Jk^b. The absence of Jk^a was confirmed by the genotyping of DNA obtained from buccal epithelial cells. The source of the Jk(a+) phenotype was traced to the transfused units. Two of these patients became immunized to Jk^a because they received RBCs that were antigen-matched based on an inaccurate phenotype due to a previous transfusion.

One patient was phenotyped as D− and genotyped as *RhD+*. Genotyping of individuals of African descent has highlighted complexities among D− individuals. *RhD* is generally absent in D− Whites carrying the *cde* haplotype. However, exceptions have been reported from Whites with the less frequent *Ce* and *cE* haplotypes and among D− individuals of African descent.[35-38] The *RhD* pseudogene (*RhDψ*), characterized by an insertion of 37 bp leading to a premature stop codon, can inadvertently

cause a discrepancy in genotype and phenotype correlation unless a specific assay[29] for detecting this insertion is employed. Although the *RhDψ* is found in D− South African (66%) and African American individuals (24%),[29] and although our study population was of African ethnicity, we did not find D− RBCs caused by this nonfunctional *RhD* by the multiplex PCR that detects *RhDψ* in the 40 SCD patients and 100 blood donors representative of this ethnicity.

The D− patient who was genotyped as *RhD*+ had anti-D in his circulation, and had been receiving D− blood, confirmed by the phenotyping performed in segments of the transfused units. Molecular analysis showed that this patient carried a partial D with four mutations: 455A>C (heterozygous), 602C>G and 667T>G (homozygous), and 1025T>C (heterozygous). These results suggest that this individual carries one *D^{IIIa}*,[30] and one *DAR*.[31] These findings, in conjunction with a previous report that *RhDψ* is of high prevalence in populations with similar background,[38] strongly suggest that genotype determination of Rh must include a thorough analysis of *RhD*. In this study, we used two multiplex PCRs, one[28] to detect gross chromosomal alterations in *RhD* and *RhCE,* including gene rearrangement and hybrid genes, and the other[29] to detect *RhDψ*. Furthermore, the multiplex PCR that detects *RhDψ* has the advantage of identifying *C* and/or *c* in the presence of *RhD* at the DNA level, a desirable feature in transfusion practice.

The contribution of genotyping to the management of SCD patients is also illustrated by the sample that was phenotyped as E+e− and genotyped as *RhEe*. The presence of *Rhe* was confirmed by genotyping DNA obtained from buccal epithelial cells. The E+e− phenotype was traced to a recently transfused unit. This patient was receiving RBC units that lacked e (R_2R_2) and was not immunized because she had both *RhE* and *Rhe*. Genotyping facilitated the management of this patient, as the prevalence of the R_2R_2 RBCs that she was previously receiving is 1 in 50. Knowing that this patient was *RhEe* allowed us to give her RBCs phenotyped as E+e− (R_2), which has a prevalence of 1 in 7, preserving resources and making more compatible units available.

The six patients previously mistyped by hemagglutination benefited from receiving antigen-matched RBCs based on genotype, as assessed by better in vivo RBC survival, increased Hb levels, and diminished frequency of transfusions.

Together, these data demonstrate the relevance of genotype determination of blood groups for the management of multiply-transfused patients with diseases such as SCD. Genotyping was also of value in the identification of suspected alloantibodies and in the selection of antigen-negative RBCs for transfusion.

As a word of caution, we should emphasize that the interpretation of genotyping results must take into account the potential for contamination of PCR-based amplification assays and the observation that the presence of a particular genotype does not guarantee expression of this antigen on the RBC membrane. The latter is illustrated by genes with a silencing mutation in a location other than that being analyzed (e.g., a point mutation in the GATA box), a gene that is silenced by an alteration of a gene encoding a protein with a modifying effect (e.g., Rh_{mod} and Rh_{null}), or the failure to detect hybrid genes.[39-42]

In conclusion, DNA typing of blood groups by PCR-RFLP in peripheral blood WBCs contributes to the management of transfusions in SCD patients by allowing determination of the true blood group genotype and the predicted phenotype of multiply-transfused SCD patients. This ensures more accurate selection of compatible donor units and is likely to prevent alloimmunization and reduce the potential for hemolytic reactions.

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