Journal of Clinical Laboratory Analysis 15:8–13 (2001)

Blood Group Genotyping in a Population of Highly Diverse Ancestry

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Accurate phenotyping of red blood cells (RBCs) can be difficult in transfusion-dependent patients such as those with thalassemia and sickle cell anemia because of the presence of previously transfused RBCs in the patient's circulation. Recently, the molecular basis associated with the expression of many blood group antigens was established. This allowed the development of a plethora of polymerase chain reaction (PCR)-based tests for identification of the blood group antigens by testing DNA. The new technologies complement phenotyping and overcome some of the limitations of hemagglutination assays. These molecular assays were developed on the basis of DNA sequences of individuals of Caucasian ancestry. The present study addresses the concern that these genotyping assays may not be applicable to populations of highly diverse ancestry because of variability in intronic regions or because of unrecognized alleles. We determined both phenotype and genotype for *RH D, K 1/K 2*, *JK A/JK B*, *FY A/ FY B*-GATA in 250 normal blood donors using PCR. Phenotype and genotype results agreed in 100% of the cases, indicating that molecular genotyping protocols can be effectively applied to populations with a highly diverse genetic background. However, genotyping for Duffy antigens provided information that could not be obtained by phenotyping. Essentially, 30.5 % of the donors with the *FY B* gene typed as Fy(b–) because of mutations in the GATA box. This information is very useful for the management of transfusion dependent patients. J. Clin. Lab. Anal. 15:8–13, 2001.

Key words: genotyping assays; blood group antigens; transfusion-dependent patients; Brazilian population; hemagglutination

INTRODUCTION

Alloantibodies to blood group antigens are clinically important in the immune destruction of red blood cells (RBCs) in allogeneic blood transfusions, feto-maternal incompatibility, autoimmune anemias, and organ transplantation (1). The detection of blood groups antigens and identification of alloantibodies by hemagglutination assays contribute significantly to blood transfusion practice and to the management of pregnancies at risk of hemolytic disease of the newborn (2). Accurate immunohematologic evaluation is critical for the safety of transfusion-dependent patients including those with sickle cell disease, thalassemia, autoimmune hemolytic anemias, and aplastic anemias. The incidence of alloimmunization to RBC antigens is particularly high in these patients (3–11). Sensitized patients receive antigen-matched RBC transfusions. In some programs, patients in need of chronic transfusion receive antigen-matched RBC transfusions in order to prevent alloimmunization. There is no consensus among different institutions about the selection of antigens negative units for the prevention of alloimmunization (12–14). In our institution matching includes, in addition to ABO, phenotyping for Rh, Kell, Duffy, and Kidd. Phenotyping of these patients and of patients that received massive transfusions can be time-consuming and hard to interpret because of the presence of donor RBCs in the recipient's circulation.

Molecular biological approaches to the study of blood group antigens have elucidated the molecular bases of most blood group alleles and phenotypes. These technologies have been applied to clinical settings and complement classical hemagglutination techniques in the clinical laboratory (15–18). However, most of the primer sets that we have developed for molecular genotyping were based on sequences of one or few individuals, usually of Caucasian origin, and in-

Grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo; Grant number: FAPESP 99/03620-0.

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Received 27 April 2000; Accepted 28 July 2000

clude at least one primer in intronic region of the gene (19–23). The present study addresses the concern that these genotyping assays may not be applicable to populations of highly diverse ancestry because: (1) at least one of the primers used in these assays is located in intronic sequences, and there may be less evolutionary pressure to preserve introns than exons; and (2) miscegenation (genetic mixture) may facilitate the generation of new alleles (24).

The Brazilian population is of heterogeneous ethnical origin. This diverse population is also unevenly distributed within a country of continental dimensions. In addition to the Native American population, Brazil is home to immigrants from Portugal, Italy, Spain, Germany, Japan, and the Middle East. During the slave trade from the 16th to the 18th century approximately 4 million Africans, mainly from Angola, Congo, and Mozambique, settled in almost all regions of Brazil (25). The intense process of miscegenation made the Brazilian population unique in its ethnic background. The region of Southeastern Brazil is the most populated, has continuously received immigrants from Northeastern Brazil, and is characterized by a high degree of admixture between descendants of Europeans, Africans, and Native Americans. Blood donors from this region constitute a representative sample of a genetically diverse population.

We found that the new molecular technologies can be safely applied to the genetically diverse population of Brazil. We also observed that, in the case of Duffy antigens, molecular genotyping can contribute substantially to transfusion management because of the high prevalence of *FY B* individuals with a mutated GATA box preventing its expression on RBCs.

MATERIALS AND METHODS

Blood Samples

The studies were performed on blood samples from 250 blood donors to the Hemocentro Unicamp. All agreed to participate in this study and signed an informed consent. The donors selected had donated at least three times in the past and been phenotyped for Rh, Kell, Kidd, and Duffy blood group antigens.

Agglutination Tests

Phenotypes were determined by hemagglutination in gel cards (Diamed AG, Morat, Switzerland) using two sources of antibodies.

DNA Preparation

DNA was extracted from blood samples using the Easy DNA Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The DNA preparations were analyzed for quality by agarose gel electrophoresis and quantified by measurement of optical density at 260 nm.

Polymerase Chain Reaction (PCR) Amplification

The primers and amplification conditions were previously described (17,18). Briefly, PCR was performed with 100–200 ng of DNA, 50 pmols of each primer, 2 nmols of each dNTP, 1.0 U *Taq* DNA polymerase and buffer in a final volume of 50 µl. The PCR profile in the thermal cycler (480, Perkin Elmer, Foster City, CA) 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 subjected to electrophoresis in 1.5% agarose gel in Tris-Acetate EDTA buffer (TAE), to verify amplification efficiency before treatment with restriction enzymes.

The PCR analysis for the presence of *RH D* was performed in two genomic regions, intron 4 and exon 10, as previously described (18). For intron 4, a primer set (RHI41/RHI42/RHI43) yielded a product of 115 bp for *RH D* and 236 bp for *RH CE*. For exon 10, a common 5′ primer (EX10F) was used for both *RH D* and *RH CE*. When paired with the *RH D*-specific 3′-untranslated region (UT) primer (RHD3UT), it produced a product of 245 bp, and when paired with the *RH CE*-specific 3′-UTR (RHCE3UT), it yielded a product of 160 bp (Fig. 1).

RFLP Analysis

Analysis of results was performed under code without knowledge of phenotype test results. 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 1x buffer according to manufacturer's instructions.

Bsm I, *Mnl* I and *Ban* I enzymes were used to determine, *K 1/K 2* (698C > T), *JK A/JK B* (838A > G) and *FY A/ FY B* (125 G > A; 265 C > T; 298 G > A) (17–20,24,26), respectively (Figs. 2, 3, 4). Furthermore, the *Sty* I enzyme was used to distinguish between normal and mutated GATA-1 binding motif (–33T > C), because the G > ATA-1 binding site is critical for Duffy protein expression in the red cell membrane (21,22,27–29). The RFLP analyses were performed after electrophoresis in 3% agarose in TAE or 8% polyacrylamide gel (PAGE) in Tris-Borate-EDTA.

RESULTS

There was complete agreement between phenotype and genotype for Rh, Kell, and Kidd in the studied population of Brazilian blood donors with highly diverse ancestry. Thus, the PCR primer sets worked adequately for the determination of genotypes despite the use of primers designed to hybridize with intronic regions of sequences obtained from Caucasians. Results are summarized in Table 1.

The genotyping approach for Rh, Kell, and Kidd was straightforward. Of the 250 blood donors samples, 207

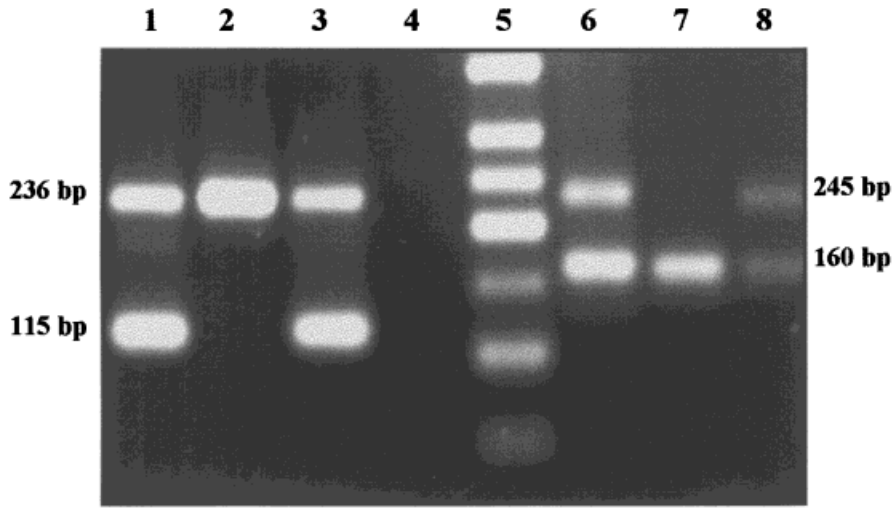

Fig. 1. Picture of 1.2% agarose gel subjected under UV after electrophoresis of PCR products. Lanes 1–3, PCR products amplified with RHI41/RHI42/RHI43 primer set; lanes 1 and 3, *RH D* positive samples 1 and 3 display bands of 236 bp for *RH CE* and 115 bp for *RH D* intron 4 sequence; lane 2, *RH D* negative sample 2 displays only the 236-bp band corresponding to *RH CE* intron 4 sequence; lane 4, reaction blank; lane 5, 50-bp DNA ladder; lanes 6–8, showing PCR products amplified with primer set EX10F/RHD3′-UTR/RHCE3′-UTR; lanes 6 and 8, *RH D* positive samples 6 and 8 display bands of 245 bp for *RH D* and 160 bp for *RH CE* exon 10 sequence; lane 7, *RH D* negative sample 7 displays only the 160 bp band corresponding to *RH CE* exon 10 sequence.

phenotyped as RhD+ and were also positive for *RH D* in both intron 4 and exon 10/3′UTR (e.g., they had amplified products for both *RH D* and *RH CE* sequences). The 43 samples phenotyped as RhD– were also genotyped as *RH D*-negative in both intron 4 and exon 10/3′UTR (e.g., they had amplified from *RH CE*, but not from *RH D* sequences) (Table 1, Fig. 1). Complete correlation between genotype and phenotype was also observed in Kell (Table 1, Fig. 2) and Kidd (Table 1, Fig. 3).

The correlation between Duffy phenotype and genotype confirmed previous observations showing that a substantial number of individuals with *FY B* genotype do not express this antigen on the surface of RBCs (Table 1). Thus, appropriate correlation between genotype and phenotype required complete analysis of the *FY* polymorphisms 125 G > A (*FY A/FY B*), 265C > T/298G > A (associated with Fy^x phenotype), and analysis of mutations in the GATA box (–33 T > C). The Duffy protein is absent from the erythroid lineage of individuals with mutated GATA box (27–29). There is a high prevalence of GATA mutation associated with the *FY B* allele. Sixty-six (30.5%) of the 216 samples with *FY B* genotype had mutated GATA box and were phenotyped as Fy(b–). Three of the donors had the phenotype Fy(b–) and the mutations associated with Fy^x (Table 1). Phenotypically, Fy^x is characterized by weak expression of *FY B* that can only be detected using potent anti-Fy^b reagent (26,30). Unfortunately, reagents capable of detecting Fy^x were not available. Fy(a–b–) were *FY B/FY B* heterozygous GATA mutation and had both 265C > T and 298G > A.

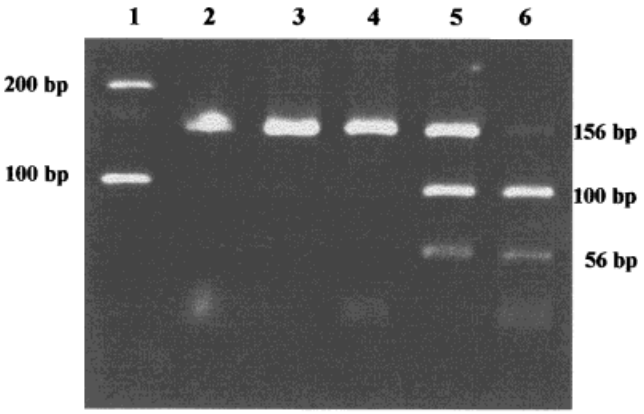

Fig. 2. Picture of RFLP analysis of PCR products amplified with KEL S/KEL R primer set using *Bsm* I after electrophoresis in 8% acrylamide gel. Lane 1, 100-bp DNA ladder; lane 2, undigested PCR product; lanes 3 and 4, samples 1 and 2, *K 2/K 2* homozygous; lane 5, sample 3, *K 1/K 2* heterozygous; lane 6, sample 4, *K 2/K 2* homozygous.

DISCUSSION

Our study demonstrated that the PCR primers for Rh, Kell, Kidd, and Duffy that were designed according to genomic sequences of individuals of Caucasian ancestry and included sequences of intronic regions could be used for blood group genotyping of populations of highly diverse ancestry. There was complete agreement between phenotype and genotype for RhD, Kell, and Kidd antigens. In the case of Duffy, 66 (30.5%) of the 216 specimens that were genotyped as *FY B* were phenotyped as Fy(b–), emphasizing the importance of the analysis of the GATA box in these populations. In the

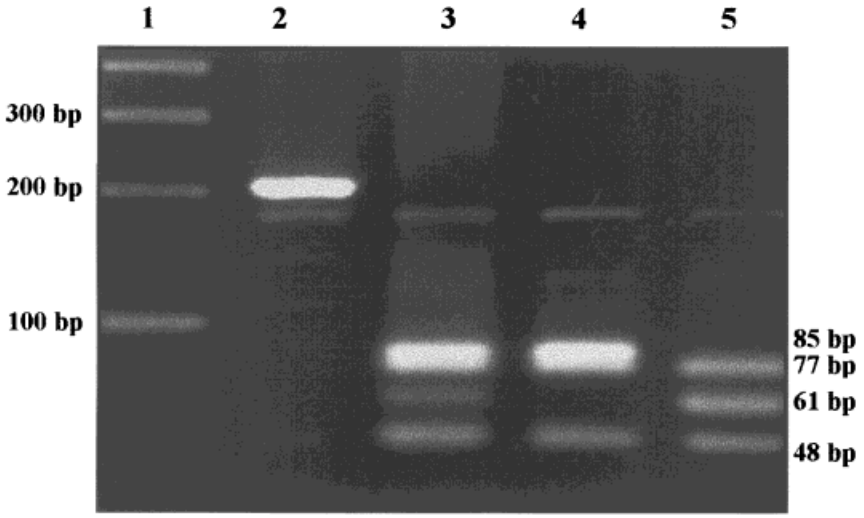

Fig. 3. Picture of RFLP analysis of PCR products amplified with JK1S/JK2 primer set using *Mnl* I after electrophoresis in 8% acrylamide gel. Lane 1, 100-bp DNA ladder; lane 2, undigested PCR product; lane 3, sample 1, *JK A/JK B* heterozygous; lane 4, sample 2, *JK B/JK B* homozygous; lane 5, sample 3, *JK A/JK A* homozygous.

presence of normal GATA-1 binding motif (nt –33 T) and normal expression of *FY B* (nt 265 C), phenotype and genotype agreed. When the GATA-1 motif is mutated, or *FY B* had Fy^x sequence (nt 265 T), a pseudo-discrepancy is observed because of the absence or weakening of FY gene expression in the erythroid lineage (26,29,30). Expression of the *FY B* gene in other body tissues is normal and these individuals do not make antibodies to Fy(b+). Thus, patients with Fy(b–) phenotype and *FY B* genotype with mutated GATA box can be transfused with Fy(b+) RBCs without the risk of alloimmunization. The combined use of genotyping and hemagglutination reduces the need for Fy(b–), a phenotype present in only one third of the Brazilian blood donors. Sixty-six (66%) of the 100 Fy(b–) donors identified in this study could theoretically receive Fy(b+) RBCs.

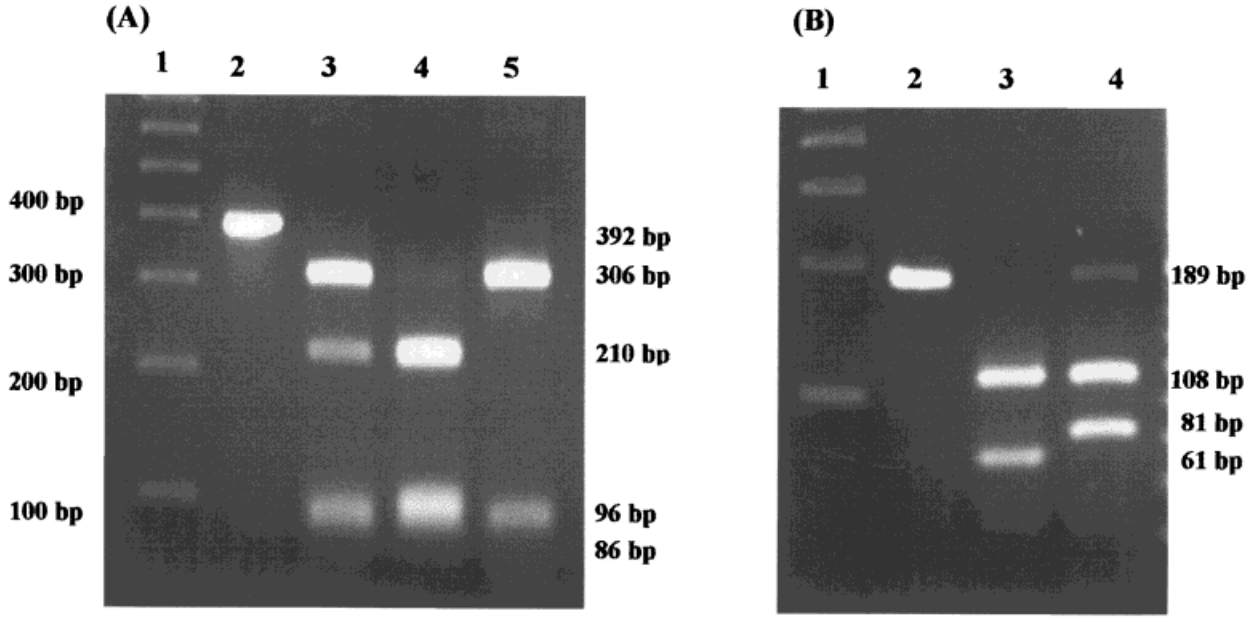

Fig. 4. **A**, RFLP analysis of PCR products amplified with FYAB1/FYAB2 primer set using *Ban* I after electrophoresis in 3% agarose gel. Lane 1, 100-bp DNA ladder; lane 2, undigested PCR product; lane 3, sample 1, *FY A/FY B* heterozygous; lane 4, sample 2, *FY A/ FY A* homozygous; lane 5, sample 3, *FY B/FY B* homozygous. **B**, RFLP analysis of PCR products amplified with FYN1/FYN2 primer set using *Sty* I after electrophoresis in 8% acrylamide gel for detection of GATA mutation. Lane 1, 100-bp DNA ladder; lane 2, undigested PCR product; lane 3, sample 1, homozygous for GATA mutation; lane 4, sample 2, homozygous wild type.

TABLE 1. Phenotyping and genotyping results for *RH D, K 1/K 2, JK A/JK B,* and *FY A/FY B-GATA* on samples from 250 blood donors

Genotype	Phenotype			
Rh system	RhD+	RhD–		
RHD+/RHCE+	207	0		
RHD –RHCE+	0	43		
Kell system	K+k+	K–k+		
K1K2	227	0		
K2K2	0	23		
Kidd system	Jk(a+b–)	Jk(a+b+)	Jk(a–b+)	
JKA/JKA	54	0	0	
JKA/JKB	0	161	0	
JKB/JKB	0	0	35	
Duffy system	Fy(a+b–)	Fy(a+b+)	Fy(a–b+)	Fy(a–b–)
FYA/FYB (T/T)	0	68	0	0
FYA/FYB (T/C)	28	0	0	0
FYB/FYB (T/T)	0	0	82	0
FYB/FYB (T/C)	0	0	0	3[a]
FYB/FYB (C/C)	0	0	0	35

[a]Fy^x 265T, 298A.

The determination of blood group polymorphism at the genomic level facilitates the resolution of clinical problems that cannot be addressed by hemagglutination. They are useful to: (1) determine antigen types for which currently available antibodies are weakly reactive; (2) type patients who have been recently transfused; (3) identify fetuses at risk for hemolytic disease of the newborn; and (4) to increase the reliability of repositories of antigen negative RBCs for transfusion.

It is important to note that PCR-based assays are prone to different types of errors than those observed with hemagglutination assays. For instance, contamination with amplified products may lead to false positive test results. In addition, the identification of a particular genotype does not necessarily mean that the antigen will be expressed on the RBC membrane. This complexity is exemplified by the existence of genes that silence mutations in locations other than that being analyzed (e.g., point mutation in the GATA box), by genes that are silenced by the a gene-encoding protein with modifying effect (e.g., Rh_{mod}, Rh_{null}), and by the detection or lack of detection of a hybrid gene (31–34).

Transfusion-dependent patients have sickle cell disease, thalassemias, and aplastic anemias, and frequently become alloimmunized. Blood group genotyping contributes substantially to the safety of blood transfusion in these recipients. Although it is unlikely that molecular genotyping will replace hemagglutination any time in the near future, together these techniques have substantial value in the resolution of clinical laboratory problems, and consequently in the quality of patient care.

ACKNOWLEDGMENTS

We thank Roberta Violati and Kim Hue Roye for technical assistence. We are also grateful to Dr. Celso Bianco for his critical review of the manuscript and to Dr. Nelson F. Mendes for his support.

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