RHD Allelic Identification Among D−Brazilian Blood Donors as a Routine Test Using Pools of DNA

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Background: RHD alleles leading to a reduced expression of D antigen of the red blood cell (RBC) surface may be erroneously typed as D− by serology and may cause anti-D immunizations when transfused to recipients. *Methods:* To determine the occurrence of such alleles among apparent D− blood donors, molecular typing was implemented as a routine test using a pool of DNA. A total of 2,450 pretyped D− samples were tested in pools of 10 for the *RHD*-specific polymorphism in intron 4 and exon 7. Samples in polymer chain reaction (PCR) positive pools were individually reevaluated by exon-specific PCRs, sequencing, and serologic methods. *Results:* Among 2,450 serologically D− blood donor samples tested, 101 (4.1%) carried the *RHD* gene. Nonfunctional *RHD* (*RHD*ψ, *RHD*CE(2–9)-D*, and *RHD*CE(3–7)-D)*, different weak D alleles such as *RHD*weak D type 1*, *RHD*weak D type 4.3*, *RHD*weak D type 5*, *RHD*weak D type 38*, and *RHD*DEL* were identified. *Conclusion:* We employed a PCR-based assay for *RHD* as a routine test using pools of ten DNA blood donor samples. The integration of *RHD* genotyping into the routine screening program using pools of DNA samples was straightforward. As a consequence, 19 (0.8%) blood donors carrying a weak D and Del phenotypes with the potential of causing anti-D immunizations in recipients were reclassified as D+. For each population, it would be necessary to adapt the *RHD* genotyping strategy to the spectrum of prevalent alleles. J. Clin. Lab. Anal. 26:104–108, 2012. © 2012 Wiley Periodicals, Inc.

Key words: pool of DNA; RHD alleles; D antigen; blood donors; PCR

INTRODUCTION

The Rh blood group system is clinically important, because antibodies against Rh antigens are involved in hemolytic disease of the newborn, hemolytic transfusion reactions, and autoimmune hemolytic anemia (1). The Rh blood group system belongs to one of the most diverse antigen systems presently known in humans and the D antigen is the most important blood group antigen determined by a protein because D-negative individuals can be easily anti-D immunized (1, 2).

Rh antigens are carried by two highly homologous proteins, the RhD and RhCE, which are encoded by two genes, *RHD* and *RHCE*, located at chromosomal position 1p34–1p36. Both genes encompass ten exons, and their structures are highly homologous (2). In previous studies, using pools of DNA, it has demonstrated that different genetic forms of D negativity occur with high incidences of unexpressed *RHD* alleles among D-negative individuals with the presence of C and/or E antigens (3–6). In general, Caucasians with the RhD-negative phenotype have the absence of the *RHD* gene due to a deletion of *RHD* between the upstream and downstream Rhesus boxes (7). However, other D-negative haplotypes exist. In African populations, some individuals who are D-negative can harbor nonfunctional *RHD* alleles, the *RHD*ψ gene and *RHD-CE(3–7)-D* hybrid (8–10). In Asians, a very weak

expression of the D antigen, the DEL phenotype, was found in individuals who were apparently D-negative and represents 10–33% of the Japanese and Chinese serologically typed as D-negative (11). Less common D-negative alleles are caused by hybrid *RHD-CE-D* alleles or nonsense and frame shift mutations (3–6).

Among Europeans, 0.6–1% carries *RHD* alleles producing weak D and partial D (12). Up to 80 weak *RHD* alleles have been described and are due to missense mutations exclusively located in the transmembranous or intracellular parts of the RhD protein (13). A small fraction of weak D samples are explained by qualitatively altered RhD proteins, called partial D and frequently caused by a *RHD/RHCE* hybrid allele (13, 14). A weak D expression is also caused by the suppressive effects of Cde haplotypes placed in trans position (15, 16).

RHD alleles leading to a reduced expression of D antigen on the red blood cell (RBC) surface may be erroneously typed as D− by standard serologic methods, including the indirect antiglobulin test (IAT) and may cause anti-D immunizations when transfused to D− recipients (17). The adsorption/elution technique considered more sensitive is not suitable for routine diagnostic use. D variants detectable by this technique are known as Del and have also caused the generation of anti-D when transfused to D-negative patients (18).

The molecular typing overcomes the limitations of serologic methods in order to solve discrepancies and to identify *RHD* variants. The *RHD* alleles are currently determined by performing PCR assays in individual samples. However, molecular typing of each sample can be time consuming, expensive, and labor intensive, representing a challenge to be performed as a routine in the clinical setting. One of the major concerns in daily practice transfusions is to prevent anti-D alloimmunization without adding much to the costs of antigen D typing and without incurring in wasting D− units unnecessarily. Therefore, a method for *RHD* genotyping that is less expensive and straightforward is highly desirable.

We developed a method herein for *RHD* typing that applies a pool of 10 DNA samples from apparently D-negative blood donors. Our lower-cost method has shown to have great sensibility/sensitivity and to be a great alternative to be used in transfusions centers.

MATERIAL AND METHODS

Blood Samples

A total of 2,450 blood donor samples from Southeast (São Paulo, SP) and Northeast (Recife, PE) Brazil labeled D− were investigated. Blood samples used for screening purposes were the by-product of blood donation and were collected according to the approved protocol of the blood banks. Whole units of blood were taken with informed consent.

Study Design and Validation of DNA Pool

The study design used to analyze the samples is shown in Figure 1.

Validation of DNA pool typing was performed by mixing nine pretyped negative DNA samples with one reference DNA sample genotyped for *RHD* variants. The sensitivity and specificity of the assay were verified by the presence of the specific amplification product, at the DNA concentration of 10ng/μL per sample.

Serologic Studies

D antigen reactivity was analyzed by agglutination in duplicate screening on an automated gel system (Wadiana®, Grifols, Derio, Spain) using two different anti-D MoAbs, one IgM (clone P3×61), and oligoclonal antibodies (clones P3×290 (IgG), P3×35 (IgG), P3×61(IgM), P3×21223B10 (IgM)) (Griffols, Derio, Spain) and on an automated microplate system (PK7200, Olympus Diagnostic Systems) using anti-D oligoclonal antibodies (clones MS26(IgG)/MS201(IgM)) (Fresenius-Kabi, São Paulo, Brazil). Nonreactive samples were tested with anti-D blend (clones MS26/MS201) using the IAT in tube. C, E, c, e status of all RBCs were determined by hemagglutination in gel neutral cards (Diamed AG, Morat, Switzerland) using routine anti-C, anti-E, anti-c, anti-e MoAbs (Fresenius Kabi, São Paulo, Brazil).

PCR Assays

Genomic DNA was individually isolated using the QI-Amp DNA Blood Mini-Kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations.

Pools of ten samples of serologically D-negative were tested with a PCR assay used to determine the presence or absence of *RHD*-specific amplified products from sequences in the intron 4 and exon 7 (10). We used 1μL of genomic DNA at the optimal DNA concentration of 10 ng/μL per sample in a final volume of 20 μL of reaction mix. When positive results were found, the pool was first divided in two and the PCR repeated in each pool of five samples.

When a five sample pool generated a PCR-positive result, each sample of the pool was tested individually in a PCR system using sequence specific primers that detect the common weak D types (19), a multiplex PCR that detects the *RHD* gene hybrid alleles (20). *RHD* zygosity was determined by checking for the presence of the hybrid Rhesus box, by an allele-specific PCR (21) and a *Pst* I-RFLP (22).
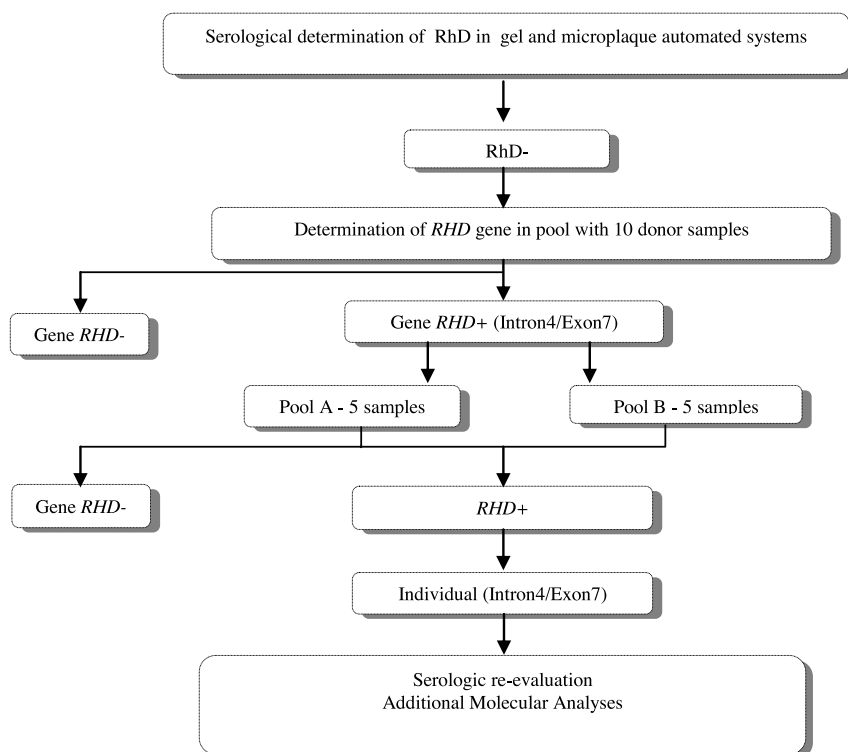
Fig. 1. Study design.

Sequence Analysis

Sequence analysis was performed on PCR products amplified from genomic DNA to determine the specific allele present using *RHD* specific primers as previously reported (23). PCR products were purified by elution from 1% agarose gels using a Qiaex II gel extraction kit (Qiagen), and sequenced directly, without subcloning, on an ABI 373XL Perkin Elmer Biosystems (PEB) sequencer, and the PEB Big Dye reagent BD Half-term (GenPak, Perkin Elmer Biosystems, Foster City, CA).

Serological Followup

Blood samples with positive results in the PCR screening were reevaluated by an IAT in two gel matrix techniques (Diamed and Grifols) using a MoAb anti-D IgG (clone ESD1). Adsorption/elution test was also performed on all nonreactive samples in IAT but positive in the PCR screening using a MoAb anti-D IgG (clone MS-26) (Fresenius-Kabi, São Paulo, Brazil). For anti-D adsorption to RBCs, 500 μL of RBCs were incubated for 1 hr at 37°C with 500 μL of anti-D diluted 1:2. Elution was performed by using the ELU–KIT II (Immucor Gamma, Houston, TX).

RESULTS

Molecular Analyses

We found 101 donors (4.1%) who carried the *RHD* gene. All 101 samples genotyped as D+ were *RHD* heterozygous. Table 1 summarizes the distribution of the *RHD* alleles found in this D− donor population, their molecular alteration detected, and the associated haplotypes.

Regional differences in the composition of the group of donors studied were encountered and reflected the

TABLE 1. Distribution of the *RHD* Alleles Identified Among D− Blood Donors, the Polymorphisms Detected, and the Associated Haplotypes

RHD allele	*N* (%)	Polymorphisms detected	Associated haplotype
RHDψ	78 (3.2)	37-bp insertion	*ce*
*RHD*CE(2-9)-D*	1 (0.04)	CE exons 2–9	*Ce trans*
*RHD*CE(3-7)-D*	3 (0.1)	CE exons 3–7	*(C)ce^s*
*RHD*DEL*	5 (0.2)	1227G>A	*Ce*
*RHD*weak D type 1*	1 (0.04)	809T>C	*Ce*
*RHD*weak D type 4.3*	5 (0.2)	602 C>G, 667 T>G, 819 G>A, 872 C>G	*ce*
*RHD*weak D type 5*	3 (0.1)	446C>A	*cE*
*RHD*weak D type 38*	5 (0.2)	833 G>A	*Ce*

TABLE 2. *RHD* **Alleles Identified Among D− Blood Donors and Serologic Results**

RHD allele	IAT/gel[a]	Adsorption/elution[b]
*RHD*ψ	−	−
*RHD*CE(3-7)-D*	−	−
*RHD*DEL*	−	+
*RHD*weak D type 1*	+	+
*RHD*weak D type 4.3*	−	+
*RHD*weak D type 5*	+	+
*RHD*weak D type 38*	+	+

[a]IAT/gel (gel cards from Diamed and Grifols) using a MoAb anti-D IgG (clone ESD1).
[b]Adsorption/elution test was performed using a MoAb anti-D IgG (clone MS-26) and the ELU–KIT II (Immucor Gamma).

diverse Brazilian genetic background. The five DEL carriers identified in this study were from Asian descent, the five weak D type 38 were from Portuguese descent, the weak D types 1 and 5 were from Caucasian descent, and the 5 weak D type 4.3 were from African descent. The RhCE phenotypes associated with weak D and *DEL* alleles were consistent with published data (24).

Serological Followup

Nine different weak D phenotypes such as weak D type 1, weak D type 5, and the weak D type 38 were identified by serologic reevaluation showing positive IAT results. Weak D type 4.3 and Del phenotypes were only detected by adsorption and elution. Table 2 shows the *RHD* alleles identified among D− blood donors and their serologic results.

DISCUSSION

Any blood bank must ensure that D-negative products are appropriately labeled, such that a recipient of a D-negative product does not form anti-D in response to transfused RBCs (1, 17). In this study, we screened serologically D- donors for the presence of the *RHD* gene and presented a fast and reliable method for *RHD* genotyping using DNA pools. We established this practice in our blood bank because current serologic practice has failed to determine weak D types (16), and to discriminate between the Del and the true D–phenotypes.

This approach revealed that 19 (0.8%) blood donors carrying weak D and Del phenotypes were mistyped as D-negative by serology, higher than the incidence of 0.4% previously reported in Caucasians (4) and comparable to the relative occurrence of 0.6 and 1% described for Germany and Canada (5, 25–27).

The presence of the nonfunctional *RHD, RHD-CE(2–9)-D, RHD-CE(3–7)-D* and the high prevalence of

*RHD*ψ (3.2%) found in this donor population reinforce that the ethnic background of the population may govern which *RH* alleles are prevalent. Our population, in fact, has ethnic characteristics related to the marriage among Caucasians, Amerindians, and Africans.

One of the major concerns in daily transfusion practice is to prevent anti-D alloimmunization, as anti-D may induce hemolytic transfusion reactions and remains the major cause of hemolytic disease of the fetus and newborn (1, 7).

A total of nine weak D phenotypes such as weak D type 1, weak D type 5, and the weak D type 38 were identified by serologic reevaluation showing positive IAT results in gel, reinforcing that the sensitivity of the method and the anti-D reagent used may influence on D donor typing result. To avoid further mistyping in our blood bank we started to use molecular techniques, as a way to avoid potential alloimmunization in the patients. However, the use of molecular techniques may represent higher costs. For this reason we investigated an alternative technique in which pools of 10 DNA samples were combined in a single reaction to decrease labor and costs of PCR reactions. By performing this pool, we were able to decrease the costs of PCR typing from approximately US\$21 to US\$13, which represents a reduction of almost 50%. Previous studies employing pools of DNA to genotype D-negative donors also showed that this strategy can contribute significantly to the total costs what is considered an acceptable strategy in terms of cost saving (5,6). Another benefit of using PCR over serological analysis is the fact that PCR genotyping usually does not need to be repeated. Few laboratories, however, are using molecular workup on a daily basis to type D antigen.

In an editorial of Transfusion, Garratty (28) stressed the caveat that not every blood donor in the D− donor pool who is carrying an *RHD* gene necessarily expresses the D antigen. Donors with the *RHD* pseudogene, for instance will not express the D antigen; however D− donor carrying aberrant *RHD* alleles leading to a reduced expression of D antigen on the RBC surface may cause anti-D immunizations when transfused to D− recipients. The overall appearance of 0.8% antigen D+ samples among D− blood donors indicates that *RHD* genotyping of D− donors could improve the RBC unit safety by permanently removing D+ donors from the D− pool and transferring their RBC units to the D+ pool as already discussed by Flegel et al. (5). According these authors, it also has the advantage that it obviates the need for a sensitive D antigen testing at the serologic level: a very strict quality assurance for sensitivity may not be required anymore, which may return some cost savings (5).

Another important benefit of using PCR is that the genotyping information can be transferred from institution to institution nationally and internationally, which

would eliminate the need for repeat testing of blood donors. The method described herein also opens the opportunity to extend DNA typing to other antigens using pools, especially when we look for rare donors. It is not unreasonable to think that donor testing in pools of DNA may also be used for typing other highly polymorphic systems, such as platelets and leukocytes.

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