Letter to the Editor

Rh D-positive genotypes in Brazilian blood donors with D-negative phenotype

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The mission of blood banks is to provide a useful community services and products of excellence in the field of hemotherapy. Thus, efforts are made to ensure that blood products are correctly identified, are free of pathogen contamination, and are available for transfusion. One of our major concerns is to prevent anti-D alloimmunization due to blood units mistyped as D-negative and to avoid hemolytic transfusion reactions. In Brazil, the technical regulations for hemotherapy procedures from the Ministry of Health require that samples with negative reactions at room temperature in donor D typing routine should be investigated for weak D by using a confirmatory test, while all Rh D variant donor samples with positive reactions are considered D-positive and the blood units are labeled as Rh D-positive (1).

Several studies have demonstrated limitations in serological testing to correctly identify the Rh D antigens of blood donors and D-positive donors have been mistyped as D-negative. To ensure safe transfusions, there is a need to identify those red blood cell units erroneously labeled as Rh D-negative.

This study aimed to confirm the serological results of blood donors typed as D-negative by molecular methods. We evaluated blood samples collected with ethylenediaminetetraacetic acid (EDTA) anticoagulant from donors of the Regional Blood Center of Ribeirão Preto, Southeast Brazil, São Paulo. During forty days (January to February 2018), 648 blood donors were phenotyped as Rh D-negative using the automated analyzer NEO (Fresenius Kabi) and selected for this study. D typing was performed by hemagglutination in a tube with two commercially anti-D monoclonal antisera (one anti-D blend clones RUM-1 and MS26 from Lorne Laboratories and one anti-D IgG clone MS26 from Fresenius Kabi) according to the manufacturer's instructions. For molecular analyses, we used DNA from a pooled blood sample extracted using a QIAamp DNA microkit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Our *RHD* genotyping strategy was initiated with DNA extracted from a twenty-blood sample pool of serologically D-negative donors. The polymerase chain reaction (PCR) assay was used to determine the presence or absence of *RHD*-specific region of intron 4 and exon 7 as previously described (2). When a twenty-sample pool generated a PCR-positive amplification for Intron4/Exon7 we deconstructed the pool and constructed four new pools containing five blood donor samples in each one. Finally, when the five-sample pool showed positive results for Intron4/Exon7, it was deconstructed and the blood samples were tested individually (*Figure 1A*). Before the study design, we validated DNA pool genotypes using nineteen *RHD* negative DNA samples and one reference *RHD* positive DNA sample. Among 648 D-negative samples studied, 17 (2.6%) carried the *RHD* gene. All 17 samples were RHD

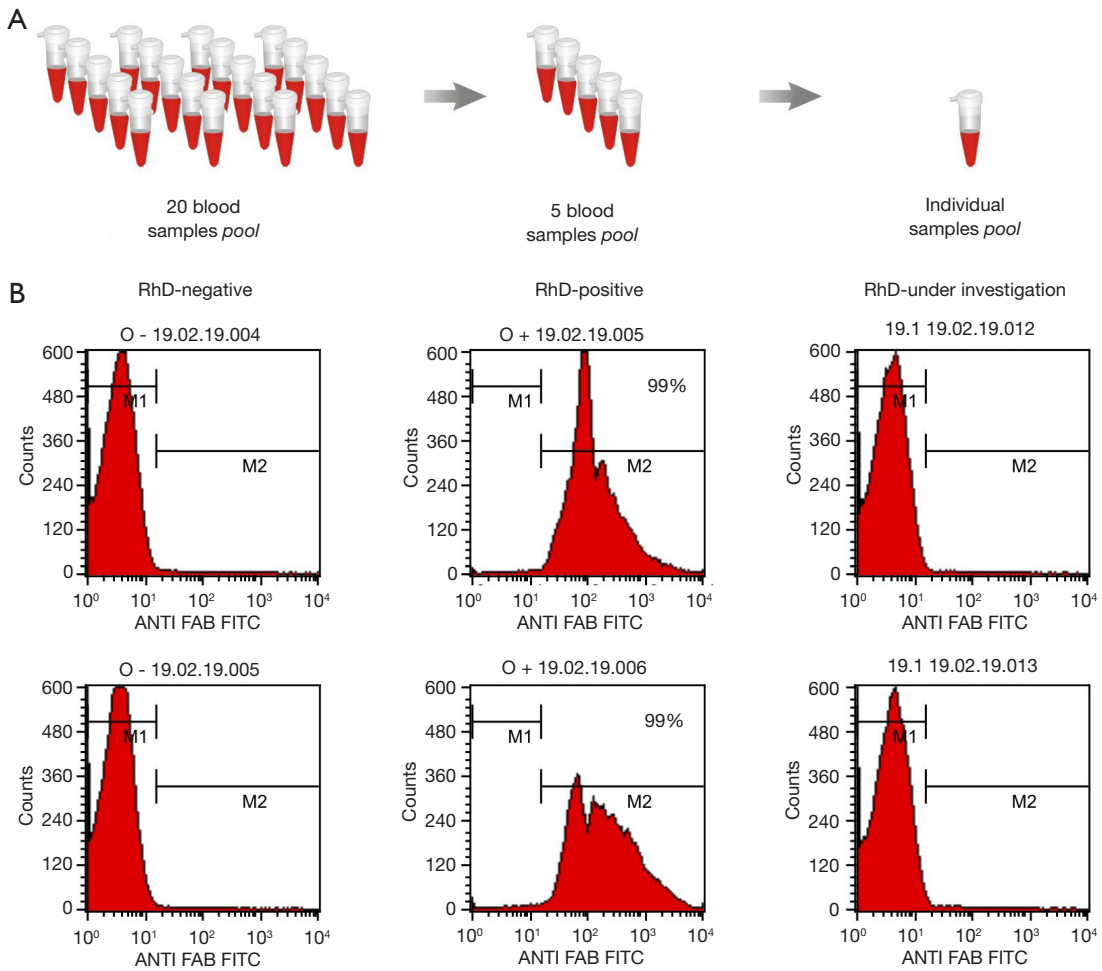

Figure 1 Study design and RhD protein analysis. (A) Experimental strategy for building blood sample *pools* for molecular analysis of the *RHD* gene. Initially, twenty blood donors' samples were pooled and the aliquot of this pool was used for DNA extraction. When a twenty-sample pool generated a PCR positive amplification for Intron4/Exon7, we deconstructed the pool and constructed four new pools containing five blood donor samples in each one. Finally, when the five-sample pool showed positive results for Intron4/Exon7, it was deconstructed and the blood samples were tested individually. (B) Expression of RhD protein in the RBC membrane. Flow cytometry analysis of the percentage of cells expressing the RhD protein. Histogram showing the percentage of cells expressing RhD; Cells were incubated with antibodies, RUM-1 and MS26 (top) and anti-D IgG clone MS26 (bottom). RBC sample obtained from D-negative donor (negative control), D-positive donor (positive control), and the sample under investigation of Rh expression. PCR, polymerase chain reaction.

hemizygous as observed by RHD zygosity testing (3). In addition, PCR-sequence specific (PCR-SSP) using primers Intron3/for2 (5'-AACCTGGGAGGCAAATGTT-3') and Intron4 insert/rev (5'-AATAAAACCCAGTAAGTTCAT GTGG-3') revealed fifteen PCR-positive amplification for *RHD* pseudogene (*RHD*$^{+}\Psi$). The two reminiscent samples followed to be evaluated using quantitative multiplex PCR of short fluorescent fragments (4), and between these samples, we found one hybrid *RHCE*ce48C-D(9)-ce*. However, one sample remained with an unconcluded molecular background. To better investigate the molecular parameters in this sample we used the Sanger sequence for the 10 exons of the *RHD* gene (5). The sequence analysis showed 100% genetic similarity in almost the 10 exons, except exon 10 which revealed a single nucleotide substitution (1347A>G) (c.*93A>G). The demographic data showed that this sample was obtained from a male donor 47 years old with a possible Afro-Brazilian genetic

background (typical African features), typed as O D–, C–, c+, E+, e+ by hemagglutination. An additional serological analysis was performed by Indirect Antiglobulin Test (IAT) in gel using the anti-D IgG monoclonal antibody (MoAb) clone ESD1 from BioRad. No Rh D antigen was detected. D-negative results were also observed in the adsorption/elution test performed using an anti-D MoAb IgG diluted 1:4 (clone MS-26) from Fresenius-Kabi. Flow cytometry results corroborated with serological results showing no evidence of RhD protein expression in the erythrocyte membrane (*Figure 1B*).

According to the literature, the substitution (c. 1347A>G) in the 3' untranslated region (3' UTR) of exon 10 was previously attributed to aberrant or non-functional *RHD* alleles (6). However further experiments have to be performed to investigate better this specific case, such as mRNA evaluation, intronic SNPs analysis, and investigation of the *RHAG* gene. As known, RBC D antigen expression is dependent on many factors, including transcriptional efficiency of the alleles, translational and trafficking of the protein to the surface, and stability of its surface expression.

There is great clinical interest in the ability to correctly identify Rh D-positive and D-negative red cells since anti-D antibodies are involved in the destruction of erythrocytes and related to serious transfusion reactions. In our study, we screened serologically D-negative donors for the presence of the *RHD* gene. The majority of donors typed as D-negative showed a complete lack of the *RHD* gene (97.4%), whereas 2.5% of the donors who carried the *RHD* gene had the non-functional RHDpsi allele (7). The presence of a D-negative RH haplotype associated with *RHDΨ* is very common in people of African origin, about 24% of African Americans and 17% of South African donors of mixed race have *RHDΨ* (7). In Brazil, it has been reported that 3.5% of blood donors typed as D-negative present the *RHDΨ* (8). Most cases of D-negative phenotype result from antigen deletion and *RHDΨ*, but the presence of hybrid genes may also be involved. One limitation of our study is related to the lack of investigation of hybrid alleles carrying smaller gene conversions in all the samples. As known, the Brazilian population's genetic miscegenation could be one factor contributing to the presence of hybrid genes and other molecular mechanisms generating RHD variants. Another study that corroborates with our findings described that 179/206 (87%) of the D-negative Brazilians entirely lacked *RHD*, 22/206 (11%) had non-functional *RHDΨ*, and 5/206 (2%) had the *RHD-CE-Ds* hybrid gene associated with the VS+V– phenotype (9). In this work, we demonstrated relevant results firstly for transfusion practice, as the successfully screened D-negative samples using sample *pools* and the identification of RHD-positive genotypes in blood donors with D-negative phenotypes from the southeastern Brazil population. The same experimental strategy of sample *pools* might be used in combination with real-time PCR analysis for *RHDΨ*, providing greater agility and security for releasing hematological results. According to previous studies, real-time PCR can be applied to screen for *RHDΨ* and a fragment of exon 5 of the wild-type *RHD* gene (10).

Strategies for genotype D-negative samples, including gene rearrangement, hybrid genes, and the *RHDΨ* in our populations are fundamental to ensure safe transfusion and to manage transfused patients. Thereby, the information provided in this study may direct an appropriate molecular assay that might be adopted in future routine immunohematology testing of donors.

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Footnote

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The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics Committee of the Regional Blood Center of Ribeirão Preto and of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil (CAAE96206818200005440), and individual consent for this study was waived.

References

1. Ministério da Saúde, Portaria número 158/2016. Technical regulation of hemotherapy procedures. Order No. 158 of February 4, 2016, BRASIL, 2016.
2. Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. BMC Genet 2001;2:10.
3. Chiu RW, Murphy MF, Fidler C, et al. Determination of RhD zygosity: comparison of a double amplification refractory mutation system approach and a multiplex real-time quantitative PCR approach. Clin Chem 2001;47:667-72.
4. Fichou Y, Le Maréchal C, Bryckaert L, et al. A convenient qualitative and quantitative method to investigate RHD-RHCE hybrid genes. Transfusion 2013;53:2974-82.
5. Legler TJ, Maas JH, Köhler M, et al. RHD sequencing: a new tool for decision making on transfusion therapy and provision of Rh prophylaxis. Transfus Med 2001;11:383-8.
6. de Paula Vendrame TA, Prisco Arnoni C, Guilhem Muniz J, et al. Characterization of RHD alleles present in serologically RHD-negative donors determined by a sensitive microplate technique. Vox Sang 2019;114:869-75.
7. 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;95:12-8.
8. Szulman A, Nardozza LM, Barreto JA, et al. Investigation of pseudogenes RHDΨ and RHD-CE-D hybrid gene in D-negative blood donors by the real time PCR method. Transfus Apher Sci 2012;47:289-93.
9. Rodrigues A, Rios M, Pellegrino J Jr, et al. Presence of the RHD pseudogene and the hybrid RHD-CE-D(s) gene in Brazilians with the D-negative phenotype. Braz J Med Biol Res 2002;35:767-73.
10. Silva-Malta MCF, Santos CCS, Gonçalves PC, et al. Molecular analysis of the RHD pseudogene by duplex real-time polymerase chain reaction. Transfus Med 2019;29:116-20.

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