Presence of the \( \text{RHD} \) pseudogene and the hybrid \( \text{RHD-CE-D}^s \) gene in Brazilians with the D-negative phenotype

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Abstract

The molecular basis for \( \text{RHD} \) pseudogene or \( \text{RHD}^\Psi \) is a 37-bp insertion in exon 4 of \( \text{RHD} \). This insertion, found in two-thirds of D-negative Africans, appears to introduce a stop codon at position 210. The hybrid \( \text{RHD-CE-D}^s \), where the 3' end of exon 3 and exons 4 to 8 are derived from \( \text{RHCE} \), is associated with the VS+V- phenotype, and leads to a D-negative phenotype in people of African origin. We determined whether Brazilian blood donors of heterogeneous ethnic origin had \( \text{RHD}^\Psi \) and \( \text{RHD-CE-D}^s \). DNA from 206 blood donors were tested for \( \text{RHD}^\Psi \) by a multiplex PCR that detects \( \text{RHD} \), \( \text{RHD}^\Psi \) and the C and c alleles of \( \text{RHCE} \). The \( \text{RHD} \) genotype was determined by comparison of size of amplified products associated with the \( \text{RHD} \) gene in both intron 4 and exon 10/3'-UTR. VS was determined by amplification of exon 5 of \( \text{RHCE} \), and sequencing of PCR products was used to analyze C733G (Leu245Val). Twenty-two (11\%) of the 206 D-negative Brazilians studied had the \( \text{RHD}^\Psi \), 5 (2\%) had the \( \text{RHD-CE-D}^s \) hybrid gene associated with the VS+V- phenotype, and 179 (87\%) entirely lacked \( \text{RHD} \). As expected, \( \text{RHD} \) was deleted in all the 50 individuals of Caucasian descent. Among the 156 individuals of African descent, 22 (14\%) had inactive \( \text{RHD} \) and 3\% had the \( \text{RHD-CE-D}^s \) hybrid gene. These data confirm that the inclusion of two different multiplex PCR for \( \text{RHD} \) is essential to test the D-negative Brazilian population in order to avoid false-positive typing of polytransfused patients and fetuses.

Introduction

The Rh blood group system is clinically important because it is involved in hemolytic disease of the newborn, hemolytic transfusion reactions and autoimmune hemolytic anemia. Rh is a highly complex red cell blood group system with 46 antigens (1,2). The most important antigens are D, C/c, and E/e. The Rh system antigens are encoded by two homologous genes (3), the \( \text{RHD} \) gene and the \( \text{RHCE} \) gene, both located on chromosome 1p34.3-p36.1 (4). \( \text{RHCE} \) gives rise to C/c and E/e polymorphism and \( \text{RHD} \) encodes the RhD antigen (5). Total or partial deletion of the \( \text{RHD} \) gene

Key words

- \( \text{RHD} \) pseudogene
- \( \text{RHD-CE-D}^s \)
- D-negative phenotype
- VS antigen
- Brazilians

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can result in the D-negative phenotype (3,6-9). In non-Whites, D-negativity can appear in individuals carrying the complete *RHD* gene (10,11). This group includes individuals of black or Asian origin (10,11) who exhibit either an internal duplication (12) or a deletion (13) within the *RHD* gene, resulting in a premature stop codon in *RHD* transcripts. The presence of certain *RHD* regions in hybrid genes encoding partial D antigens may predict a D-negative phenotype, and the presence of some *RHD* regions in genes encoding no D antigen may predict a D-positive phenotype. In order to avoid these complications, methods which detect more than one region of *RHD* have been introduced (11,14,15).

About two-thirds of D-negative Africans have an inactive *RHD* gene (12). This pseudogene (*RHDΨ*) has a 37-bp insert in exon 4, which may introduce a reading frame shift and premature termination of translation and a translation stop codon in exon 6 (12). Of the remaining one-third of African D-negative donors, about half appear to be homozygous for an *RHD* deletion and about half have the *RHD-CE-D*′ hybrid gene characteristic of the (C)e′c′ haplotype that produces c, VS, and abnormal C and E, but not D (8,12). In D-negative African Americans and South African people of mixed race, the same three genetic backgrounds are present, but 24% of African Americans and 17% of South African donors of mixed race have *RHDΨ*, and 54% of African Americans and 81% of South African donors of mixed race have no *RHD* (12).

In the present study we investigated whether D-negative Brazilian blood donors of heterogeneous ethnic origin had altered *RHD*. We studied DNA samples from 206 D-negative blood donors (50 of Caucasian descent and 156 of African descent) by two different multiplex PCR that detect *RHD*, D variants, *RHC/c* and the *RHDΨ* and by sequencing exon 5 of *RHCE* for the 733 C>G polymorphism (VS antigen). Our observation was in agreement with previous publications showing that *RHD* was deleted in all individuals of Caucasian descent. However, 14% of D-negative Brazilians of African descent studied had the *RHDΨ* and 3% had the *RHD-CE-D*′ hybrid gene. These data show the necessity of performing multiplex PCR for detecting more than one region of *RHD* and the 37-bp insertion in populations of African descent for predicting the D phenotype from DNA in order to avoid false-positive typing of polytransfused patients and fetuses.

**Material and Methods**

**Blood donors**

We studied peripheral blood samples from 206 random D-negative blood donors (50 of Caucasian descent and 156 of African descent) who agreed to participate in this study by signing an informed consent form. The study was approved by the Medical Ethics Committee of UNICAMP and CONEP.

**Agglutination tests**

RhD phenotypes were determined by hemagglutination in gel cards (Diamed AG, Morat, Switzerland) using two different commercial sources of monoclonal antisera (Gamma Biologicals Inc., Houston, TX, USA; Diamed AG). VS and V phenotypes were determined by standard techniques using polyclonal antibodies (patient serum).

**DNA preparation**

DNA was extracted from blood samples using the DNAzol (Gibco BRL, Rockville, MD, USA) and a blood DNA purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to manufacturer recommendations.

Allele-specific PCR for *RHD* genotyping-PCR analysis for the presence of *RHD* was
performed in two genomic regions, intron 4 and exon 10. Briefly, PCR was performed with 100-200 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 (9700, Perkin Elmer, Foster City, CA, USA) and the same profile was used for both assays, as follows: 15 min at 95°C, 35 cycles of 40 s at 94°C, 40 s at 62°C, and 1 min at 72°C, followed by 10 min at 72°C. Amplified products were analyzed by electrophoresis in 1.5% agarose gel in Tris-acetate EDTA buffer. For exon 10, a common 5' primer (EX10F) was used for both *RHD* and *RHCE*. When paired with the RHD-specific 3'-untranslated region (UTR) primer, it produced a product of 210 bp, and when paired with the RHCE-specific 3'-UTR primer, a product of 163 bp (16) was produced. A set of three primers, RHI41 and RHI42 (previously reported; 16), and an additional third primer RHI43 were used for intron 4. The combination of these three primers generates products of 115 bp for *RHD* and 236 bp for *RHCE* (Figure 1). The sequences of the primers are listed in Table 1.

**Multiplex PCR for the presence of the RHD pseudogene**

Analysis of the *RHDΨ* 37-bp insert was performed using a multiplex PCR that detects the presence of D, differentiates *RHCEc* and identifies *RHDΨ* (12). PCR primers are listed in Table 2. Thirty cycles of PCR were performed at 94°C for 1 min, 65°C for 1 min, and 72°C for 3 min 30 s. PCR products were analyzed by 2% agarose gel electrophoresis (Figure 2B).

**Multiplex PCR for RHD variants**

Analysis of *RHD* variants was performed in all samples using an *RHD* multiplex assay directed at six regions of *RHD* (exons 3-7 and exon 9), covering all exons with *RHD*-specific sequences in the coding regions (15). The multiplex PCR was performed in a thermal cycler (9700, Perkin Elmer) with the following cycle specifications: 32 cycles of 1 min at 95°C, 1 min at 55°C and 45 s at 72°C, followed by 10 min at 72°C. PCR products were size-separated by 8% acrylamide gel electrophoresis (Figure 2A). PCR primers are listed in Table 3.

**Sequence analysis**

Sequence analysis was performed on PCR products amplified from genomic DNA using *RHCE*-specific primers for exon 5 (Table 4) to determine the presence of 733G predicted to encode Val245 (VS+) and *RHD*-specific primers for exon 3 (Table 4) to determine the presence of the D-CE hybrid. PCR products were purified on 1% agarose gels using a Qiaex II gel extraction kit (Qiagen, Valencia, CA, USA), and sequenced directly using an ABI 373XL Perkin Elmer Biosystems sequencer.

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**Table 1. Primers used for RHD genotyping.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Intron/exon/bp</th>
</tr>
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<tbody>
<tr>
<td>RHI41</td>
<td>5'-GTG TCT GAA GCC CTT CCA TC-3'</td>
<td>Intron 4/236/115</td>
</tr>
<tr>
<td>RHI42</td>
<td>5'-GAA ATC TGC ATA CCA CAC GC-3'</td>
<td></td>
</tr>
<tr>
<td>RHI43</td>
<td>5'-ATT AGC TGG GCA TGG TGG TG-3'</td>
<td></td>
</tr>
<tr>
<td>EX10F</td>
<td>5'-TTT CCT CAT TTG GCT GTT GGA TTT TAA-3'</td>
<td>Exon 10/210/163</td>
</tr>
<tr>
<td>RH3-UTR</td>
<td>5'-GTA TCC TCT CTG ACC TAA TAA ATG GTG-3'</td>
<td></td>
</tr>
<tr>
<td>RHCE3-UTR</td>
<td>5'-CTG TCT CGT ACC TTG TTT CAT TAT AC-3'</td>
<td></td>
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</table>

**Table 2. Primers used for identification of RHD-CE-Ds.**

<table>
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<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Exon/bp</th>
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</thead>
<tbody>
<tr>
<td>Exon 7 for</td>
<td>5'-AGC TCC ATC ATG GGC TAC AA-3'</td>
<td>Exon 7/95</td>
</tr>
<tr>
<td>Exon 7 rev</td>
<td>5'-ATT GCC GGC TCC GAC GGT ATC-3'</td>
<td></td>
</tr>
<tr>
<td>Intron 3 for 1</td>
<td>5'-GGG TTG GCT GGG TAA GCT CT-3'</td>
<td></td>
</tr>
<tr>
<td>Intron 4 rev</td>
<td>5'-GAA CCT CCT CTG TGA AGT GCT-3'</td>
<td></td>
</tr>
<tr>
<td>Exon 4 insert</td>
<td>5'-AAT AAA ACC AGT AAC TTC ATG TGG-3'</td>
<td>Exon 4 37-insert/250</td>
</tr>
<tr>
<td>Intron 3 for 2</td>
<td>5'-AAC CTC GGA GGC AAA TGT TT-3'</td>
<td></td>
</tr>
<tr>
<td>C for</td>
<td>5'-CAG GGC CAC CAC CAT TTG AA-3'</td>
<td>Exon 2/320</td>
</tr>
<tr>
<td>C rev</td>
<td>5'-GAA CAT GCC ACT TCA CTC CAG-3'</td>
<td></td>
</tr>
<tr>
<td>C c for</td>
<td>5'-TGC GCC AAG ATC TGA CCG-3'</td>
<td></td>
</tr>
<tr>
<td>C c rev</td>
<td>5'-TGA TGA CCA CCT TCC CAG G-3'</td>
<td>Exon 2/177</td>
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alloanti-D reagents that react with all known partial D and weak D antigens. Red blood cells from five black donors who were D-negative were phenotyped as C+c+E-e+ VS+ and V-. These five donors all showed a weak expression of C.

Screening D-negative donors for exon 10 and intron 4

All D-negative donors were tested by the allele-specific PCR method designed to determine the presence of RHD exon 10 and intron 4 (Figure 1). Three patterns of reaction were apparent: presence of both RHD regions, absence of both RHD regions, and presence of RHD exon 10, but absence of RHD intron 4. Of the 206 D-negative Brazilian blood donors tested, 87% lacked RHD (50 of Caucasian descent and 129 of African descent), 11% had both regions of RHD, and 2% had only RHD exon 10 (Table 5).

Screening D-negative donors for the RHDΨ 37-bp insert

Of 156 D-negative samples from people of African origin, 22 (14%) contained the exon 4/37-bp insert (Table 5, Figure 2B).

Screening D-negative donors for RHD exons 3-7 and exon 9

Multiplex PCR to detect D variants (Figure 2A) in selected donors with RHD revealed that donors with RHD exon 10 and intron 4 also had RHD exons 3, 4, 5, 6, 7, and 9, suggesting the presence of a grossly intact RHD. Red cells from five donors of African descent with RHD exon 10, but without RHD intron 4, were C+ and VS+V-. In addition to RHD exon 10, donors of this type had RHD exon 9 and a hybrid exon 3 comprising a 5' end derived from RHD and a 3' end derived from RHCE. The presence of the 773G mutation in exon 5 of the RHCE determined by sequencing confirmed the VS antigenicity.
This suggests that these five donors (2%) have the RHD-CE-D gene associated with the (C)ce' complex (RHD-CE-D^p) (Table 5).

Donors with neither exon 10 nor intron 4 of RHD also lacked RHD exons 3, 4, 5, 6, 7 and 9.

**Genomic DNA analysis by sequencing**

Genomic DNA analysis performed by sequencing revealed in five donors of African descent the presence of the D-CE hybrid exon 3 and the 733G mutation [predicted to encode Val245 (VS+)], associated with the RHD-CE-D^s hybrid gene (Table 5).

**Discussion**

There are actually three genetic mechanisms associated with the D-negative phenotype: deletion of RHD (3), an RHD pseudogene containing a 37-bp insert and one or two stop codons (12), and a hybrid RHD-CE-D^s gene that probably produces an abnormal C antigen but does not produce a D antigen (8,12). RHD is generally absent in RHD-negative Caucasians carrying the cde haplotype. However, exceptions have been reported among Caucasians with the less frequent Ce and cE haplotypes and among D-negative individuals of African descent (10,14,17,18). The RHD^Ψ, characterized by an insertion of 37 bp leading to a premature stop codon, can inadvertently cause discrepancy in genotype/phenotype correlation unless a specific assay (12) for detecting this insertion is employed. RHD^Ψ is found in D-negative South Africans (66%) and in African Americans (24%) (12). In our study, 11% of the 206 D-negative Brazilians studied had this nonfunctional RHD.

An RHD-CE-D fusion gene, in which the 3' end of exon 3 plus exons 4-8 is derived from RHCE, is sometimes associated with a D-negative phenotype in people of African

<table>
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<th>Donors</th>
<th>Phenotype</th>
<th>Genotype</th>
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<tr>
<td></td>
<td>RHD^-</td>
<td>RHD^Ψ</td>
</tr>
<tr>
<td>Caucasian</td>
<td>50 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>African</td>
<td>129 (83%)</td>
<td>22 (14%)</td>
</tr>
<tr>
<td>Total</td>
<td>206</td>
<td>179 (87%)</td>
</tr>
</tbody>
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Table 5. Results of testing for the presence or absence of RHD, RHD^Ψ and RHD-CE-D^s in 206 Brazilian blood donors.

![Figure 2](image_url)
origin (8,12). The hybrid gene carries a Leu245Val substitution responsible for the VS antigen and is associated with the presence of a weak C. We found this hybrid gene in five donors of African descent phenotyped as D-Cweak c+e+E-e+VS+V-. These samples were D-positive by exon 10 analysis but D-negative by intron 4 and exon 7 analysis. The five samples were all heterozygous for C733G in exon 5 of RHCE which predicts a Leu245Val (VS antigen) and so had a probable ce/(C)ce genotype.

These findings, taken together with a previous report that RHDPΨ is of high prevalence in populations of similar background (10), strongly suggest that genotype determination of RH must include a thorough analysis of RHDP. In the present study, we used two multiplex PCR, one (15) to detect gross chromosomal alterations in RHDP and RHCE including gene rearrangement and hybrid genes, and the other (12) to detect RHDPΨ. Furthermore, the multiplex PCR that detects RHDPΨ has the advantage of determining C/c at the DNA level in the presence of RHDP, a feature that is desired in transfusion practice and to predict the RhD blood type of a fetus in populations of African descent. Typing the fetus for the RH allele is also valuable because anti-G may be responsible for hemolytic disease of the newborn.

The most common D-negative Rh haplotype in Africans is RHDPΨ with the ce allele of RHCE. The 37-bp insert in exon 4 of RHDPΨ is a duplication of a sequence spanning the boundary of intron 3 and exon 4. This insert may introduce a reading frame shift and a translation stop codon at position 210. However, the duplication introduces another potential splice site at the 3’ end of the inserted intronic sequence in exon 4 and another stop codon in exon 6 of the gene (12). RHDP mRNA was not detected in D-negative individuals with RHDPΨ, despite the presence of RHCE transcripts. In fact, Africans with RHDPΨ are truly D-negative since they can produce anti-D and cause hemolytic disease of the newborn as previously reported (12).

Our results confirm the necessity to perform multiplex PCR including gene rearrangement and hybrid genes and the RHDPΨ in populations of African descent for the appropriate management of transfused patients and for RhD-negative pregnant women who are sensitized, particularly when the fetal RHDP is determined by molecular assays.

Finally, the 11% prevalence of RHDPΨ suggests a high degree of admixture of individuals of African descent in the Brazilian population.

Acknowledgments

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References


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