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Presence of the *RHD* pseudogene and the hybrid *RHD-CE-D^s* gene in Brazilians with the D-negative phenotype

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Abstract

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

Key words
- *RHD* pseudogene
- *RHD-CE-D^s*
- D-negative phenotype
- VS antigen
- Brazilians

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 *RHD* gene and the *RHCE* gene, both located on chromosome 1p34.3-p36.1 (4). *RHCE* gives rise to C/c and E/e polymorphism and *RHD* encodes the RhD antigen (5).

Total or partial deletion of the *RHD* gene

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 (*RHD*Ψ) 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*^{s} hybrid gene characteristic of the *(C)ce*^{s} 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 *RHD*Ψ, 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 *RHD*Ψ 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 *RHD*Ψ and 3% had the *RHD-CE-D*^{s} 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 95ºC, 35 cycles of 40 s at 94ºC, 40 s at 62ºC, and 1 min at 72ºC, followed by 10 min at 72ºC. 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 *RHC/c* and identifies *RHDΨ* (12). PCR primers are listed in Table 2. Thirty cycles of PCR were performed at 94ºC for 1 min, 65ºC for 1 min, and 72ºC 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 95ºC, 1 min at 55ºC and 45 s at 72ºC, followed by 10 min at 72ºC. 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.

Table 1. Primers used for *RHD* genotyping.

Primers	Sequence	Intron/exon/bp
RHI41	5'-GTG TCT GAA GCC CTT CCA TC-3'	Intron 4/236/115
RHI42	5'-GAA ATC TGC ATA CCC CAG GC-3'	
RHI43	5'-ATT AGC TGG GCA TGG TGG TG-3'	
EX10F	5'-TTT CCT CAT TTG GCT GTT GGA TTT TAA-3'	Exon 10/210/163
RHD3'-UTR	5'-GTA TTC TAC AGT GCA TAA TAA ATG GTG-3'	
RHCE3'-UTR	5'-CTG TCT CTG ACC TTG TTT CAT TAT AC-3'	

Table 2. Primers used for identification of *RHD-CE-D*^{s}.

Primers	Sequence	Exon/bp
Exon 7 for	5'-AGC TCC ATC ATG GGC TAC AA-3'	Exon 7/95
Exon 7 rev	5'-ATT GCC GGC TCC GAC GGT ATC-3'	
Intron 3 for 1	5'-GGG TTG GCT GGG TAA GCT CT-3'	Intron 4/498
Intron 4 rev	5'-GAA CCT GCT CTG TGA AGT GCT-3'	
Exon 4 insert	5'-AAT AAA ACC AGT AAG TTC ATG TGG-3'	Exon 4 37-insert/250
Intron 3 for 2	5'-AAC CTG GGA GGC AAA TGT TT-3'	
C for	5'-CAG GGC CAC CAC CAT TTG AA-3'	Exon 2/320
C rev	5'-GAA CAT GCC ACT TCA CTC CAG-3'	
c for	5'-TCG GCC AAG ATC TGA CCG-3'	Exon 2/177
c rev	5'-TGA TGA CCA CCT TCC CAG G-3'	

Results

Serology

Red blood cells from the 206 blood donors gave D-negative results with two 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.

Table 3. Primers used for identification of the *RHDΨ* 37-bp insert.

Primers	Sequence	Exon/bp
MR364	5'-TCGGTGACTGATCTCAGTGGA-3'	Exon 3/111
MR474M	5'-ACTGATGACCATCCTCATGT-3'	
MR496	5'-CACATGAACATGATGCACA-3'	Exon 4/126
MR621	5'-CAAACTGGGTATCGTTGCTG-3'	
MR648	5'-GTGGATGTTCTGGCCAAGTT-3'	Exon 5/157
Mrex5	5'-CACCTTGCTGATCTTACC-3'	
MR898	5'-GTGGCTGGGCTGATCTACG-3'	Exon 5/57
Mrex6	5'-TGTCTAGTTTCTTACCGGCAAGA-3'	
MR973	5'-AGCTCCATCATGGGCTACAA-3'	Exon 7/96
MR1068	5'-ATTGCCGGCTCCGACGGTATC-3'	
Mre9SD2	5'-AACAGGTTTGCTCCTAAATATT-3'	Exon 9/71
MR1219	5'-AAACTTGGTCATCAAAATATTTAACCT-3'	

Table 4. Primers used for sequence analysis of VS and D-CE hybrid.

Primers	Sequence	Exon/bp
RHCEint4 sense	5'-GAG GTT GCA GTG AGC CCA TGA TCG-3'	Exon 5/474
RHCEex5 reverse	5'-TGA CCC TGA GAT GGC TGT CA-3'	
RHDex3 sense	5'-TCG GTG CTG ATC TCA GTG GA-3'	Exon 3/116
RHDex3 reverse	5'-GAT ATT ACT GAT GAC CAT CCT-3'	

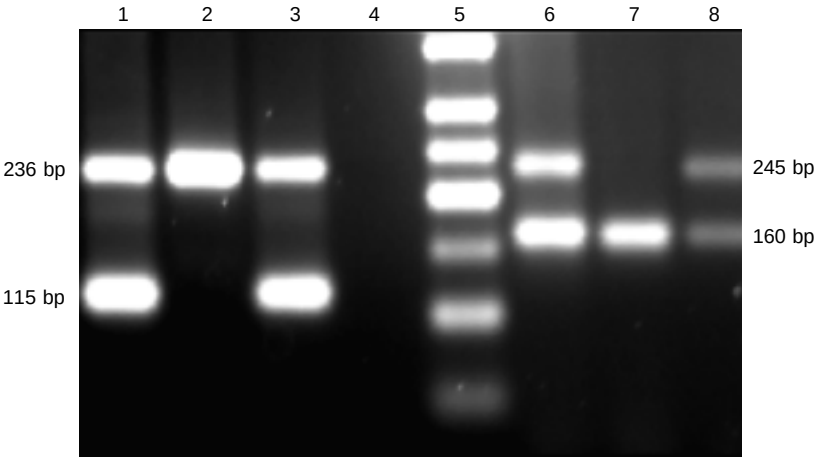

Figure 1. *RHD* genotyping by intron 4 and exon 10 of *RHD*. *Lanes 1 and 3*: *RHD*-positive samples display bands of 236 bp for *RHCE* and 115 bp for the *RHD* intron 4 sequence. *Lane 2*: *RHD*-negative sample displays only the 236-bp band corresponding to the *RHCE* intron 4 sequence. *Lane 4*: Reaction blank. *Lane 5*: 50-bp DNA ladder. *Lanes 6 and 8*: *RHD*-positive samples that amplify a 245-bp product of the *RHD* and a 160-bp product of the *RHCE* exon 10 sequence. *Lane 7*: *RHD*-negative sample displays only the 160-bp band corresponding to the *RHCE* exon 10 sequence.

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*^{s} complex (*RHD-CE-D*^{s}) (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 *RHDΨ*, 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. *RHDΨ* 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 5. Results of testing for the presence or absence *of RHD*, *RHDΨ* and *RHD-CE-D*^{s} in 206 Brazilian blood donors.

Donors	Phenotype	Genotype		
		RHD-	*RHDΨ*	*RHD-CE-D*^{s}
Caucasian	RhD-	50 (100%)	0	0
African	RhD-	129 (83%)	22 (14%)	5 (3%)
Total	206	179 (87%)	22 (11%)	5 (2%)

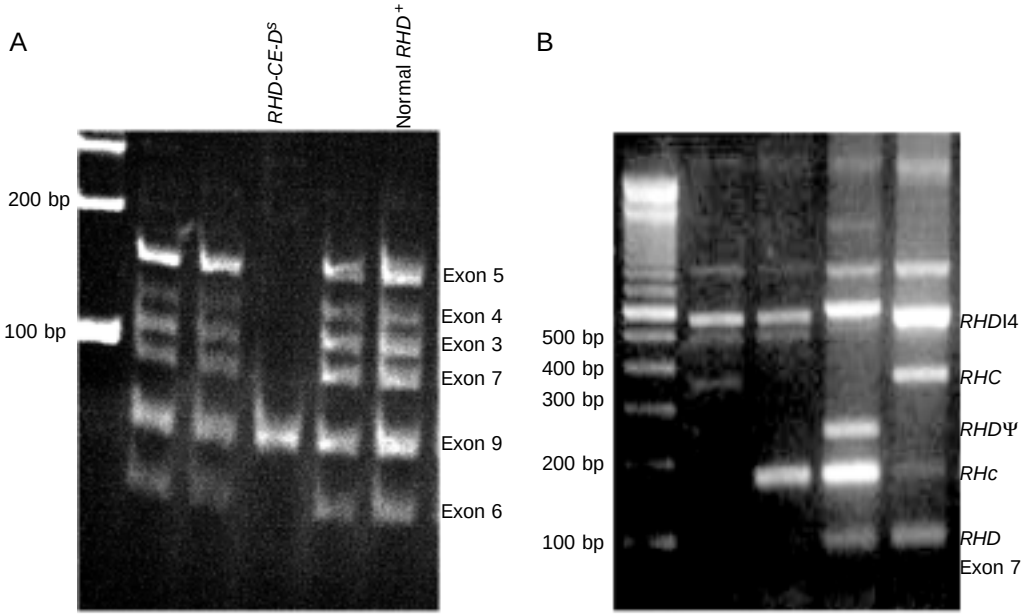

Figure 2. Multiplex PCR for detection of the *RHD* hybrid alleles and the *RHDΨ*. *A*, 8% acrylamide gel showing results with multiplex PCR products amplified with six *RHD*-specific primer sets (exons 3, 4, 5, 6, 7 and 9). *B*, 2% agarose gel showing results with multiplex PCR for predicting D and C/c phenotype and for detecting the presence of *RHDΨ*.

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-C^{weak}c+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*^{s} genotype.

These findings, taken together with a previous report that *RHD*Ψ is of high prevalence in populations of similar background (10), strongly suggest that genotype determination of *RH* must include a thorough analysis of *RHD*. In the present study, we used two multiplex PCR, one (15) to detect gross chromosomal alterations in *RHD* and *RHCE* including gene rearrangement and hybrid genes, and the other (12) to detect *RHD*Ψ. Furthermore, the multiplex PCR that detects *RHD*Ψ has the advantage of determining C/c at the DNA level in the presence of *RHD*, 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 *RHC* 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 *RHD*Ψ with the *ce* allele of *RHCE*. The 37-bp insert in exon 4 of *RHD*Ψ 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). *RHD* mRNA was not detected in D-negative individuals with *RHD*Ψ, despite the presence of *RHCE* transcripts. In fact, Africans with *RHD*Ψ 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 *RHD*Ψ 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 *RHD* is determined by molecular assays.

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

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