Weak D types 38 and 11: determination of frequencies in a Brazilian population and validation of an easy molecular assay for detection

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Recent evidence shows that, among Brazilians, the distribution of weak D types significantly differs from that represented in people of European descent, with a high percentage of weak D types 38 and 11. Our goal was to determine the population frequencies of weak D types 38 and 11 in a Brazilian population and to validate a molecular approach to identify these two variants. Blood donors were sequentially enrolled in the study in a 5-year period. Donors with serologic weak D phenotype had the *RHD* coding region sequenced. The frequencies of weak D type 38 and weak D type 11 (CDe-associated) were calculated. Two allele-specific–polymerase chain reaction (AS-PCR) assays were designed to detect *RHD*weak D type 38* and *RHD*weak partial 11* and were validated with samples positive and negative for these two variants, respectively. A total of 618,542 donors were enrolled, of which 265 presented with a serologic weak D phenotype. When considering all donors evaluated, the frequencies of weak D types 38 and 11 were 0.013 and 0.002 percent, respectively. In the subgroup of donors with a serologic weak D phenotype, the frequencies of weak D types 38 and 11 were 30.2 and 4.9 percent, respectively. The two proposed AS-PCR assays for detection of *RHD*weak D type 38* and *RHD*weak partial 11* showed 100 percent accuracy. The frequencies of weak D types 38 and 11 among Brazilians are high compared to that previously described for other populations. The AS-PCR assays to detect *RHD*weak D type 38* and *RHD*weak partial 11* represent potentially helpful tools for investigating Brazilian individuals with these weak D phenotypes. ***Immunohematology* 2020;36:47–53.**

Key Words: *RHD,* weak D, blood donors, weak D type 38, weak D type 11

D antigen is very important in transfusion practice because of its high immunogenicity and involvement with post-transfusion hemolytic reactions and hemolytic disease of the fetus and newborn.[1] The *RHD* locus is complex, and diverse gene variations (point mutations, insertions, deletions, and gene conversions) can affect the RhD phenotype and lead to variant antigens.[2–4] RhD variants may explain red blood cell (RBC) alloimmunization in the case of partial antigens or can result in misclassification of D+ individuals as D– in the presence of weak antigens.[2]

Weak D variants are characterized by a decrease in D antigen expression.[5] In general, a serologic weak D phenotype is defined by a reactivity of RBCs with anti-D reagent resulting in no (0) or weak (≤2+) reactivity in direct agglutination testing but exhibiting significant agglutination with anti-human globulin.[6] At the molecular level, weak D variants exhibit missense mutations affecting the transmembrane or intracellular domains of the D protein.[5] Approximately 0.2 to 1 percent of white individuals inherit an *RHD* allele that codes for a serologic weak D phenotype[5,6] and, in this population, these variant alleles most frequently encode weak D types 1, 2, or 3, which can be managed safely as D+.[5,7]

It is important to highlight the fact that the distribution and diversity of RhD variants vary according to population, race, and geographical location. Most of what is known about RhD variants stems from studies focusing either on a population of European descent or on patients with sickle cell disease.[8–10] It has been recently suggested that weak D types 1, 2, and 3 represent less than 30 percent of the serologic weak D phenotypes identified among Brazilians, who are intensely racially mixed.[11] Relatively high frequencies of weak D type 38 and weak D type 11 in this population were also shown,[11,12] which is an interesting finding, since these variants are infrequent among white individuals and not reported among people of African ancestry.

Based on this information, our main goal was to determine the frequencies of weak D types 38 and 11 in a Brazilian population and to standardize an easy molecular method to detect both variants, which could be implemented into the routine of blood banks that perform molecular blood group analysis.

Materials and Methods

Donor Recruitment and Serologic Tests

Donors for evaluation presented sequentially at the blood center (Fundação Pró-Sangue Hemocentro de São Paulo) from October 2014 to August 2019. The study was approved by the local ethics committee and followed the Helsinki principles.

Participant samples were tested for D between October 2014 and September 2018 using the microplate hemagglutination method (NEO, Immucor, GA). Samples giving negative results were further tested using a solid-phase method (Capture Solid-Phase Technology, Immucor). For both microplate hemaggutination and solid phase, anti-D clones D145 (IgM) and D415 (IgG) were used. From October 2018 to August 2019, donor samples were tested for D using the gel method (anti-D clones ESD-1M + 175-2) and the IH-1000 instrument (Bio-Rad, Cressier, Switzerland).

Samples were selected for *RHD* genotyping if they met the following criteria: (1) reactivity less than 2+ with anti-D in the gel method or (2) a negative reaction with anti-D in the microplate hemagglutination method but a positive reaction in the solid-phase test.

Considering the selection criteria for genotyping the samples, only *RHD*weak partial 11* associated with CDe haplotype and presenting with a Del phenotype would be detected. This variant differs from *RHD*weak partial 11* associated with cDe haplotype, which presents a much higher D antigen density.[5]

Nucleic Acid Purification

Genomic DNA was individually isolated and purified using the PureLink Genomic Kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Purity and concentration were evaluated by spectrophotometry (Nanodrop1000 Spectrophotometer, Wilmington, DE). DNA samples were diluted to a final concentration of 100 ng/μL for *RHD* genotyping.

RHD Genotyping

Samples were selected for *RHD* genotyping according to the serologic criteria described previously. For all selected samples, the following tests were performed: (1) amplification of *RHD* exons 3–7 and 9 in a multiplex reaction designed to detect some D-variant genotypes, as described elsewhere,[13] and (2) *RHD*Psi, RHC, RHc, RHE,* and *RHe*[14,15] genotyping. Direct sequencing (Sanger method) of the 10 exons of *RHD* (and short flanking introns) using gene-specific primers was performed for all samples in which all *RHD* exons tested (3–7 and 9) were amplified, following previously published protocols.[16] *RHD* zygosity was determined for samples exhibiting *RHD* variant alleles by two different conventional PCR-based assays designed to detect the *RHD* deletion.[17,18]

Validation of the Allele-Specific–Polymerase Chain Reaction (AS-PCR) Assays Designed to Detect *RHD*Weak D Type 38* and *RHD*Weak Partial 11*

Two AS-PCR assays were designed. The first aimed to amplify a 243-bp product of the *RHD*weak D type 38,* containing the 833G>A (G278A) mutation; the second aimed to amplify a 204-bp product of the *RHD*weak partial type 11,* containing the 885G>T (M295I) mutation. The sequences of the primers used in both assays are shown in Table 1, and the samples selected for validation are described in Table 2.

Table 1. Primers used for AS-PCR assays

AS-PCR	Primer name	Primer sequence (5´–3´)	Product size (bp)
*RHD*weak D type 38*	833G>A_F	TGCGGTGTTGGCAGGAGA	243
	833G>A_R	ATAAGAGAATGCGCCGACAC	
*RHD*weak partial 11*	885G>T_F	TCTCCGTGGCTTGCCATT	204
	885G>T_R	GGCGTTGAAGCCAATAAGAG	
Internal control	HGH_F	TGCCTTCCCAACCATTCCCTTA	434
	HGH_R	CCACTCACGGATTTCTGTTGTGTTTC	

AS-PCR = allele-specific–polymerase chain reaction; F = forward; R = reverse; HGH = human growth hormone.

As an internal PCR control, all assays contained primers for the amplification of human growth hormone (HGH), named as internal control (IC) in Figure 1.

Both PCRs were run on a Mastercycler EP Gradient 96-well thermal cycler (Eppendorf, Hamburg, Germany) in a total volume of 30 μL. The final concentrations of DNA and primers (forward and reverse) in the master mix were 100 ng/μL and 50 pmol/μL, respectively. PCR conditions were as follows: 15 minutes at 95°C, 35 cycles of 20 seconds at 95°C, 20 seconds at 60°C, and 20 seconds at 72°C, followed by 10 minutes at 72°C. PCR fragment products were subjected to electrophoresis in 2 percent agarose gel. The following bands could be observed according to the donor genotype: 243-bp PCR product for *RHD*weak D type 38,* 204-bp PCR product for *RHD*weak partial 11,* and 434-bp product for HGH (IC) (Fig. 1).

Table 2. Samples selected for the validation of AS-PCR assays to detect *RHD*weak D type 38* and *RHD*weak partial 11*

RHD genotype	Number of tested samples	Results of the AS-PCR assays	
		*RHD*weak D type 38*	*RHD*weak partial 11*
*RHD*weak D type 38/RHD*01N.01*	7	Positive	Negative
*RHD*weak D type 38/RHD*weak D type 38*	5	Positive	Negative
*RHD*weak D type 38/RHD*DAR*	1	Positive	Negative
*RHD*weak partial 11/RHD*01N.01*	8	Negative	Positive
*RHD*01/RHD*01*	2	Negative	Negative
*RHD*weak D type 1/RHD*01N.01*	1	Negative	Negative
*RHD*weak D type 2/RHD*01N.01*	1	Negative	Negative
*RHD*weak D type 3/RHD*01N.01*	1	Negative	Negative
*RHD*weak D type 5/RHD*weak D type 5*	1	Negative	Negative
*RHD*DIIIc/RHD*01N.01*	1	Negative	Negative
*RHD*DAR/RHD*DAR*	1	Negative	Negative
*RHD*DIVa/RHD*01N.01*	1	Negative	Negative
Total	30		

AS-PCR = allele-specific–polymerase chain reaction.

To determine the accuracy of the proposed assays, samples known to exhibit *RHD*weak partial 11* or *RHD*weak D type 38,* as determined by direct Sanger sequencing, were tested. Samples with other *RHD* variants and with conventional *RHD* were also tested to detect potential false-positive results.

Determination of D Antigen Density

The D antigen density of samples with predicted weak D type 38 and weak D type 11 phenotypes was determined by flow cytometry (FACSCalibur; Becton Dickinson, Heidelberg, Germany) according to a previously described protocol[19] and using commercial monoclonal anti-D (IgG) clone ESD1 (DiaMed Latino America S.A., Lagoa Santa, Brazil). Two samples testing as D+ (agglutination strength of 4+ with anti-D using gel method) and presenting with CcDEe phenotype were selected as positive controls. One D– sample (ccddee) was selected as a negative control.

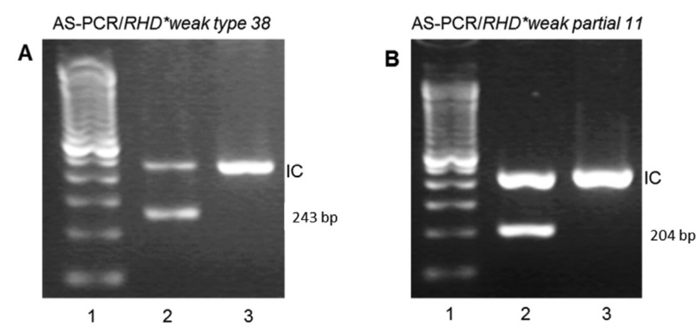

Fig. 1 Allele-specific–polymerase chain reaction (AS-PCR) assays designed to detect *RHD*weak D type 38* and *RHD*weak partial 11.* (A) AS-PCR to detect *RHD*weak D type 38.* 1 = ladder (100 bp); 2 = donor presenting with *RHD*weak D type 38* (243 bp); and 3 = donor presenting with conventional *RHD.* (B) AS-PCR to detect *RHD*weak partial 11.* 1 = ladder (100 bp); 2 = donor presenting with *RHD*weak partial 11* (204 bp); and 3 = donor presenting with conventional *RHD.* The human growth hormone internal control (IC) band (434-bp) is shown in both A and B.

Results

Frequencies of *RHD*Weak D Type 38* and *RHD*Weak Partial 11* in a Brazilian Donor Population

A total of 618,542 donors from Sao Paulo, in Southeast of Brazil, were enrolled in the study. Among the donors with anti-D results of ≤2+ agglutination strength in the gel method or negative at direct hemagglutination and only reacting by solid-phase method (n = 265), there were 80 individuals with predicted weak D type 38 phenotype (30.2%) and 13 individuals with predicted weak D type 11 phenotype (4.9%), as shown in Table 3. The calculated population frequencies of weak D types 38 and 11 were 13:100,000 (0.013%) donors and 2:100,000 (0.002%) donors, respectively.

Description of Study Donors with *Weak D Types 38* and *11* Phenotypes

The self-declared racial background of the donors with weak D type 38 phenotype was 66.25 percent white (n = 53),

Table 3. *RHD* genotype of Brazilian donors with weak D types 38 and 11 phenotypes

RHD genotype				
Allele 1	Allele 2	Phenotype	*N* (%)	Self-declared race (*n*)
*RHD*weak D type 38 (RHD*01W.38)*	Deleted D *(RHD*01N.01)*	73 R_1r and 1 R_1R_0	73 (78.4)	49 White 19 Mixed 2 Black 3 NP
*RHD*weak D type 38 (RHD*01W.38)*	*RHD*weak D type 38 (RHD*01W.38)*	R_1R_1	4 (4.3)	3 White 1 Mixed
*RHD*weak D type 38 (RHD*01W.38)*	*RHD*Psi (RHD*08N.01)*	R_1R_0	2 (2.1)	1 White 1 Mixed
*RHD*weak D type 38 (RHD*01W.38)*	*RHD*weak D type 4.0 (RHD*09.03.01)*	R_0R_0	1 (1.1)	1 Black
*RHD*weak partial 11 (RHD*01W.11)*	Deleted D *(RHD*01N.01)*	R_1r	13 (14)	10 White 2 Mixed 1 Black
Total			93	62 White (66.6%) 23 Mixed (24.7%) 4 Black (4.3%) 4 NP (4.3%)

NP = not provided.

26.25 percent mixed (*n* = 21), and 3.75 percent black (*n* = 3) (Table 3). For the donors with weak D type 11 phenotype, 76.9 percent self-declared as white (*n* = 10), 15.4 percent self-declared as mixed (*n* = 2), and 7.7 percent self-declared as black (*n* = 1) (Table 3).

In 91.25 percent of the cases with weak D type 38 (*n* = 73), *RHD*weak D type 38* was *in trans* to deleted D and, in 0.05 percent of the cases (*n* = 4), *RHD*weak D type 38* was present in the homozygous state. There were two cases in which *RHD*weak D type 38* was *in trans* to *RHD*Psi* and one case of compound heterozygosity *RHD*weak D type 38/ RHD*weak D type 4.0*. This last case represented the only situation in which *RHD*weak D type 38* was associated with the cDe haplotype, and the genotype was determined by direct sequencing of the 10 *RHD* exons (c.602C>G, c.667C>G, and c.819C>G in heterozygosity). In all other cases, *RHD*weak D type 38* was associated with the CDe haplotype.

In all cases of weak D type 11, *RHD*weak partial 11* was found *in trans* to deleted D and associated with CDe haplotype.

Validation of AS-PCR Protocol to Detect *RHD*Weak D Type 38* and *RHD*Weak Partial 11*

Thirty samples were genotyped in parallel using both the proposed AS-PCR assays and conventional Sanger sequencing. Both 833G/A (*RHD*weak D type 38*) and 885G/T (*RHD*weak partial 11*) polymorphisms were evaluated.

For *RHD*weak D type 38,* 13 samples presenting the allele and 17 samples without the allele were tested with the proposed AS-PCR assay (Table 2). The obtained genotypes were concordant with the results from Sanger sequencing, and the accuracy of the assay was considered as 100 percent.

For *RHD*weak partial type 11,* 8 samples presenting the allele and 22 samples without the allele were tested with the respective AS-PCR assay. Again, all resulting genotypes were in accordance with that obtained from Sanger sequencing, and the accuracy of the assay was 100 percent.

Determination of D Antigen Density

Four samples with weak D type 38 and one sample with weak D type 11 were studied. The mean D antigen density for weak D type 38 was 65 sites/RBC, varying from 40 to 90 sites/ RBC. In the case of weak D type 11, the calculated D antigen density was 13 sites/RBC.

Discussion

This study aimed to determine the frequencies of weak D types 38 and 11 in a Brazilian population, which is racially mixed. The calculated frequencies of these variants were 0.013 percent for weak D type 38 and 0.002 percent for weak D type 11, taking into account a total of 618,542 Brazilian donors of all RhD phenotypes. This study also standardized a simple and highly accurate molecular assay to identify *RHD*weak D type 38* and *RHD*weak partial 11,* which can be applied in the routine testing of Brazilian blood banks to easily identify these two variants.

The higher-than-expected prevalence of weak D type 38 among Brazilians has been previously suggested by some studies, which focused their analyses on samples with low expression of D.[11,12,20,21] The frequency of this D variant significantly differed from that previously calculated for individuals of European descent.[22] In fact, in some large cohorts of European individuals screened for weak D phenotype, this variant was not encountered.[5,23] Referring to weak D type 11, the frequencies in literature vary because of an increase in the sensitivity of serologic methods for D testing, and, consequently, the ability to detect this variant has also increased over time.[23,24] The population frequency of weak D type 11 associated with the CDe haplotype in southwestern Germany was 0.015 percent, higher than that found in our cohort (0.002 percent).[24]

Weak D type 38 was first observed among white individuals with weak D phenotype.[22] Even though weak D type 2 is the most common type of weak expression of D among Portuguese individuals,[25] a significant number of weak D type 38 individuals has been previously observed in this population.[26] The prevalence of weak D type 38 among Brazilians might be explained by the pivotal participation of the Portuguese population in Brazilian history.[26] To date, the frequency of this variant reported in Brazil is the highest in the literature. Considering the low-antigen density associated with this variant, applying highly sensitive serologic methods, such as gel or solid-phase, or using *RHD*-PCR to detect weak D type 38 in routine blood donor testing, is fundamental in Brazilian territory.

Even though the Brazilian population is highly mixed and the accuracy of self-reported race in predicting the actual racial ancestry is questionable, in the present study, most of the donors presenting with weak D types 38 and 11 self-declared as white (66.25% and 76.9%, respectively). The fact that weak D type 38 has not been detected in cohorts of individuals of African descent is important to highlight.[8] Weak D type 11 has been already described in the North African Tunisian population,[27] probably reflecting the strong European influence on this territory. In our results, 30 percent of the donors with weak D type 38 and 23.3 percent of the donors with weak D type 11 self-declared as black or mixed, reinforcing the racial mixing of the Brazilian population.

Considering the prevalence of weak D types 38 and 11 among Brazilians, a conventional molecular assay designed to detect both variants would be useful to assess individuals with weak D phenotype. The AS-PCR assays validated in the present study represent effective and low-cost protocols to evaluate individuals with weak D expression and R_1 phenotype. The suggested workflow to investigate donors with serologically weak D phenotype in which the validated assays are applied is displayed in Figure 2.

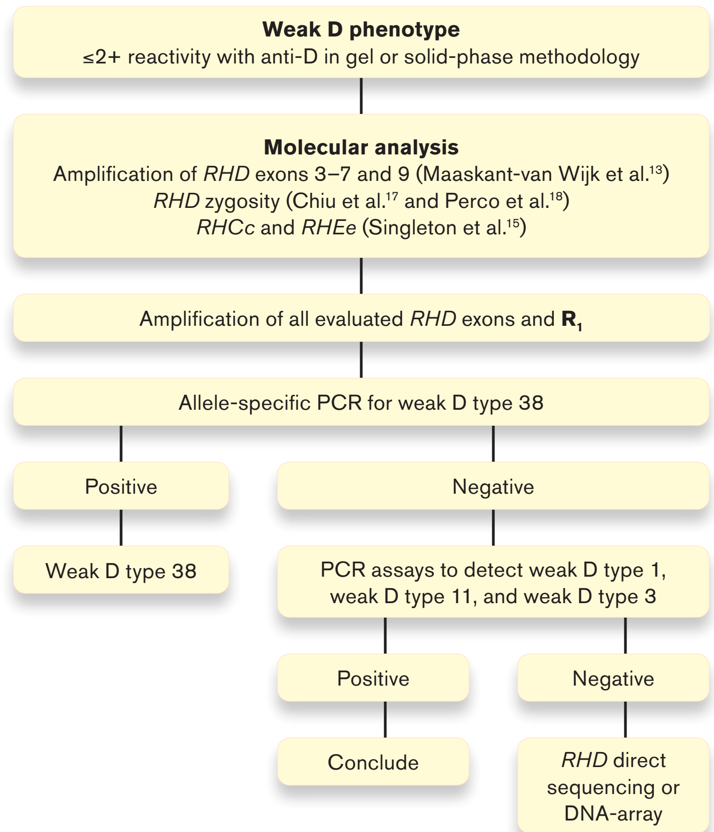

Fig. 2 Workflow used for the molecular investigation of Brazilian donors with serologic weak D phenotype. Earlier information from molecular analysis studies is incorporated.[15,17,18] PCR = polymerase chain reaction.

Finally, in our results, *RHD*weak D type 38* was mostly associated with the CDe haplotype, in accordance with previous reports in the literature. However, in one donor, *RHD*weak D type 38* was found in association with the cDe haplotype, which has not been previously described. In this specific case, the donor also presented the *RHD*weak D type 4.0 in trans* to the *RHD*weak D type 38* and self-reported as black. *RHD*weak partial 11* was associated with CDe in all cases in which this allele was detected, reflecting a biased selection of samples for genotyping. In our study, only samples weakly reacting (≤2+ agglutination strength) in the gel method or only reacting by solid phase were genotyped. As a consequence, weak D type 11 associated with the cDe

haplotype could not be detected, since it would be easily demonstrable in the indirect agglutination test.[5]

This study may be limited by the fact that blood donors included in the study were recruited from the southeastern part of the country. Considering the geographic dimensions of Brazil, the results may not reflect the reality of the entire country. The southeastern region of Brazil is the most populous, however, and previous studies investigating *RHD* variants in individuals from different parts of the country did not reveal relevant regional differences.[28] Also, as mentioned before, the selection criteria limited the detection of weak D type 11 associated with the cDe haplotype.

In conclusion, the frequencies of weak D types 38 and 11 among Brazilians are high in comparison with frequencies previously described for other populations. Because of the low-antigen density associated with these variants, highly sensitive serologic or molecular methods should be applied to the donor D phenotyping routine. Conventional molecular methods to detect *RHD*weak D type 38* and *RHD*weak partial 11* represent potentially helpful tools in the investigation of Brazilian individuals with serologic weak D phenotype and the CDe haplotype.

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