Molecular structures identified in serologically D– samples of an admixed population

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BACKGROUND: The D– phenotype is mainly caused by the complete deletion of the *RHD* gene in Caucasians. However, a plethora of allelic variants have been described among D– individuals from different ethnic groups.

STUDY DESIGN AND METHODS: A cohort of 1314 routine serologically D– samples from white Argentineans was studied by molecular methods.

RESULTS: Among the 1314 D– samples, 2.1% showed *RHD*-specific amplifications. One hybrid *Rhesus box* was detected in all D–/*RHD*+ samples, suggesting a hemizygous status. The *RHDΨ* was found in 0.7% of rr samples while *DEL* and *null* variants were detected in 16.7% of the D– samples expressing C and/or E antigens. The variants associated with the C antigen were seven *RHD-CE-D^s*, two *RHD(1-2)-CE(2-9)-D(10)*, two previously unreported *RHD(329T>C)-CE(3-9)-D* null alleles, one *RHD(M295I)*, and one new *RHCE(1-2)-RHD($3_{361del11}$-10)* null allele whereas those associated with the E antigen were five *RHD(46T>C)* and one novel *RHD(581insG)* null allele responsible for a premature stop codon.

CONCLUSIONS: The prevalence of D–/*RHD*+ samples is higher than that observed in Europeans. More than 50% of the *RHD* alleles found were represented by *RHDψ* and *RHD-CE-D^s* showing the African contribution to the genetic pool of the admixed population analyzed. Interestingly, three new alleles were found, two of them being hybrid structures between previously described *RHD* variants recombined with *RHCE* sequences. The knowledge of the *RHD* allele repertoire in our population allowed the implementation of reliable typing and transfusion strategies for a better management of patients and pregnant women.

The Rh polypeptides are encoded by more than 250 variant forms of the two highly homologous *RHD* and *RHCE* genes. The D antigen, carried by the RhD protein, is by far the most clinically important because of its high immunogenicity and is still one of the leading causes of hemolytic disease of the newborn. This antigen is also involved in posttransfusion alloimmunization events. In almost 100% of D– Caucasians, the absence of the D antigen is caused by the complete deletion of the *RHD* gene. However, a plethora of allelic variants have been described among D– individuals from different ethnic groups. While *RHDψ* and *RHD-CE-D^s* alleles are carried by approximately 66 and 16% of D– Africans, respectively, in Europeans the hybrid *RHD-CE(2-9)-D* is the most frequent silent variant found in 0.2% to 0.6% of D–/*RHD*+ individuals.[1-4]

Approximately 30 *RHD* alleles are responsible for a significantly reduced expression of the D antigen on red

ABBREVIATIONS: SSP = sequence-specific priming; UTR = untranslated region.

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blood cells (RBCs). This so-called DEL phenotype is not detected by standard serology even when D typing is performed with a sensitive indirect antiglobulin test (IAT). Therefore, DEL RBCs are frequently mistyped as D– unless adsorption-elution tests are performed. Although rare in Europeans, *RHD(M295I)* and *RHD(IVS3+1G>A)* seem to be the most frequent alleles found. In contrast, up to 30% of seemingly D– blood donors in East Asia carry the *RHD(1227G>A)* allele.[2,5,6] It is still controversial if the detection of DEL is of any clinical relevance; however, some cases of anti-D immunization by DEL RBCs have been reported.[7,8]

The current population of Argentina is the result of generations of intermixing that started around the 1500s between Native Amerindians, Spanish conquerors, and Africans brought as slaves. Later, the massive immigration from Spain and Italy at the beginning of the 20th century made the European genetic component and physical appearance predominant nowadays. More recently, further admixture events have been introduced by immigration from other South American countries.[9,10] Therefore, the complex process of immigration and admixture in Argentina has left an imprint in the genetic composition of this country that is scarcely known in many fields. In this sense, a comprehensive study of the *RHD* polymorphism occurring in our population would help to determine the most suitable DNA typing scheme that could be used to appropriately classify D status in patients and pregnant women. In this work, we have investigated the molecular basis of the D– phenotype in individuals from the city of Rosario. We have been able to identify *RHD null* and *DEL* variants and to describe novel *RHD* alleles within the group of D–/*RHD*+ individuals. The results obtained have also given some insight into the genetic variability of our population.

MATERIALS AND METHODS

Samples

A total of 1314 D– EDTA-anticoagulated blood samples were randomly selected for this study. Samples belonged to a cohort of unrelated individuals from the city of Rosario, Argentina. For all samples written informed consent was obtained according to the Declaration of Helsinki.

Serologic analysis

The D antigen status of each sample was evaluated by hemagglutination with a commercially available monoclonal-monoclonal blended anti-D (Wiener Lab, Rosario, Argentina) containing immunoglobulin (Ig)M clone TH-28 and IgG clone MS-26. When an immediate spin–negative result was observed, the samples were tested by the IAT. The anti-D used reacts with most weak D and partial D RBCs, including DVI, in the IAT. D– samples were also tested with anti-C (Clone MS24), anti-c (Clone MS33), anti-E (Clone MS80 + MS258), and anti-e (Clones MS16 + MS21 + MS63; Rediar, Buenos Aires, Argentina) and entered the workflow for molecular characterization. All standard serologic tests were performed in tube according to the manufacturer's instructions.

Molecular studies

In a first step, D– samples were analyzed for the presence of Intron 4 and the 3′ untranslated region (UTR) of the *RHD* gene using a multiplex polymerase chain reaction (PCR) strategy as described previously.[11] All samples were also screened in parallel for *RHD*-specific sequences encompassing part of the 5′ UTR and Exon 1 by PCR with sequence-specific priming (SSP) using a forward primer specific for G at Position –58 (5′-TTCCGTGTTAAC TCCATAGAG-3′) and a reverse primer specific for G at Position 48 (5′-GCTTCCAGTGTTAGGGCC-3′). This amplification was performed with approximately 50 ng of genomic DNA in a final volume of 10 µL containing 0.2 µmol/L each primer (except for internal control primers used at 0.1 µmol/L to coamplify a 434-bp fragment from the human growth hormone gene), 0.2 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI) in appropriate buffer. PCR procedures started with one cycle of denaturation at 94°C for 2 minutes and followed with 30 cycles of 10 seconds at 94°C, 40 seconds at 58°C for annealing, and 40 seconds at 72°C for extension. PCR products were analyzed by electrophoresis on 2% agarose gels stained with DNA gel stain (SYBR Safe, Life Technologies, Carlsbad, CA). A 229-bp product indicates the presence of the *RHD* specific 5′ UTR.

RHD zygosity of all samples containing *RHD*-specific fragments was evaluated by amplification of the downstream and hybrid *Rhesus boxes* followed by digestion of the PCR products with endonuclease *Pst*I. An additional restriction site that is only present in the hybrid *Rhesus box* (5275G) produces a 564-bp fragment diagnostic for the *RHD* gene deletion.[12] In all rr samples containing *RHD*-specific fragments the *RHDψ* allele was investigated by PCR.[13]

The rest of the D–/*RHD*+ samples were subjected to an *RHD* exon scanning procedure based on PCR-SSP to analyze multiple *RHD* exon polymorphisms.[14,15] When *RHD* Exons 3 to 7 were not amplified, the *RHD-CE-D*^{s} allele was investigated by a specific PCR that amplifies a *5′RHD-RHCE3′* hybrid Exon 3 characteristic of the Type 1 variant.[16] On the other hand, in all *RHD-CE(3-9)-D* hybrid structures, the *Taq*I Intron 1 restriction fragment length polymorphism (RFLP), located approximately 1 kb upstream of Exon 2 and the Intron 2 *Pst*I RFLP found approximately 3100 bp downstream of Exon 2 were

investigated as described.[17,18] Also, the DNA region encompassing Exon 2 was sequenced as previously reported.[19]

Uncharacterized samples were subsequently analyzed using the microarray-based BLOODchip platform (Progenika Biopharma, Vizcaya, Spain) that allows the identification of more than 100 *RHD* alleles. Briefly, PCR-amplified *RHD* regions were fragmented, labeled with a fluorescence marker, and hybridized to probes attached to the surface of a glass slide. Fluorescence intensity was detected by a confocal scanner, quantified, and analyzed on proprietary software to determine the genotype and predicted phenotype.[20]

In the absence of known alleles, direct automated sequencing on PCR products of the 10 *RHD* exons, adjacent intronic sequences and 5′ promoter region, was performed.[19]

Serologic and molecular follow-up

Samples carrying the *RHD(46T>C)* allele were reevaluated with different anti-D (IgM Clone MS-201, IgM Clone RUM-1, and IgM Clones LDM1 + ESD1M) and anti-E (IgM Clone MS-260, IgM Clone MS-80) reagents. Adsorption-elution assays were also performed with a polyclonal anti-D obtained from a sensitized individual. The *RHCE* gene was sequenced in all *RHD(46T>C)* samples as previously reported.[21] D antigen expression of the sample carrying the *RHD(581insG)* allele was evaluated by an adsorption-elution assay.

The presence of *RHD* Exon 1 and Exon 2 of the sample that failed to amplify the *RHD* 5′ UTR region was investigated with primer pairs specific for intronic *RHD* polymorphisms as described.[19] Intron 1 and Intron 2 polymorphisms were determined as mentioned as well.

RESULTS

Among the 1314 D– samples studied, 1200 (91.3%) rr phenotypes were encountered while C and/or E antigen expression was found in 114 (8.7%) individuals. *RHD*-specific amplifications were detected in 27 of the 1314 D– samples. The amplification patterns, Rh phenotypes, and *RHD* alleles found are shown in Table 1.

One hybrid *Rhesus box* was detected in all D–/*RHD*+ samples suggesting a hemizygous status for the *RHD* locus.

Interestingly, the infrequent *RHD(46T>C)* allele was found by sequencing in five unrelated r″r patients. Further serologic characterization using an extended set of anti-D clones and adsorption-elution assays confirmed an extremely weak expression of the D antigen in all samples carrying this mutation (data not shown). Moreover, in two of these samples an altered E antigen expression was also detected with anti-E IgM Clones MS-80 and MS80 + MS258 associated with the *RHcE(697C>G, 712A>G, 733C>G, 744T>C)* allele (data not shown). The cis position of both *RHD(46T>C)* and *RHcE(697C>G, 712A>G, 733C>G, 744T>C)* variants was assessed by family studies that could be performed in one of the probands (Fig. 1).

As a first approach to determine the 5′ breakpoint region of the *RHD-CE(3-9)-D* hybrid structures encountered in four r′r phenotypes, the first informative polymorphisms found upstream and downstream of Exon 2 were investigated. The results obtained in the RFLP analysis demonstrated the *RHD*-specific sequence in Intron 1 and the *RHC*-specific sequence in Intron 2. These results do not allow accurately defining the *RHD* or *RHC* origin of Exon 2 and suggest that the recombination site is within the 4.2 kb homology region located between both polymorphisms. Surprisingly, Exon 2 sequence analysis detected the 329T>C mutation, characteristic of the partial *DVII* allele, in two of the samples, suggesting that in these cases the new hybrid structure found could be the result of homologous recombination between a *DVII* allele and *RHCE* sequences. This variant was dubbed *RHD(329T>C)-CE(3-9)-D*. The alleles found in the other two samples most likely represent the already described *RHD-CE(2-9)-D* variant (Fig. 2A).

TABLE 1. *RHD* alleles in serologically D– samples

RHD fragments	*RHD* allele	CE phenotype	Number of samples (n = 1314)	%
5′ UTR: –	Deletion	ccee	1192	
Intron 4: –	Deletion	Ccee	63	
3′ UTR: –	Deletion	ccEe	25	97.9
	Deletion	CcEe	6	
	Deletion	ccEE	1	
5′ UTR: +	*RHDΨ*	ccee	8	
Intron 4: +	*RHD(46T>C)*	ccEe	5	
3′ UTR: +	*RHD(M295I)*	Ccee	1	
	RHD(581insG)	ccEe	1	
5′ UTR: +	*RHD-CE-D^{s}*	Ccee	7	2.1
Intron 4: –	*RHD-CE(2-9)-D*	Ccee	2	
3′ UTR: +	*RHD(329T>C)-CE(3-9)-D*	Ccee	2	
5′ UTR: –	*RHCE-D($3_{361del11}$-10)*	Ccee	1	
Intron 4: +				
3′ UTR: +				

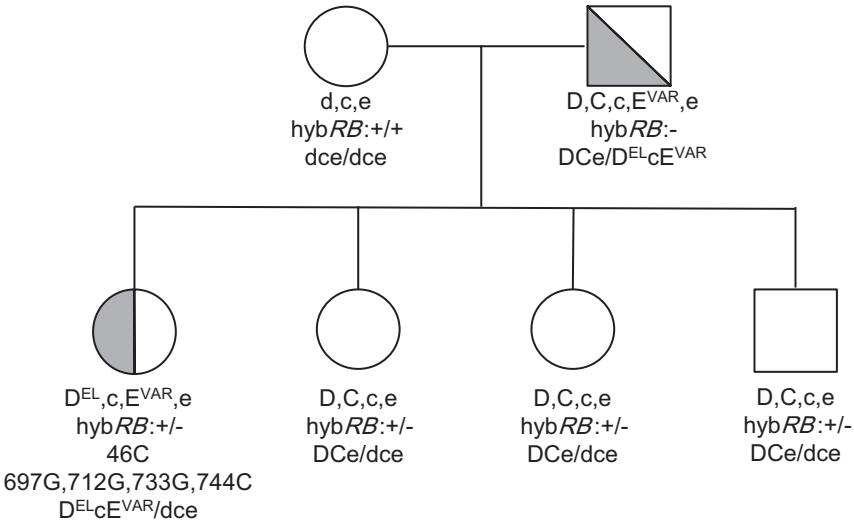

Fig. 1. Serologic, molecular, and segregation analyses of a DEL and altered E expression. Rh phenotype, the presence of one (+) or two (+/+) hybrid *Rhesus boxes* (hyb*RB*), *RHD* and *RHCE* polymorphisms, and haplotypes are shown. Gray color indicates that both *RHD(46T>C)* and *RHcE(697C>G, 712A>G, 733C>G, 744T>C)* alleles are present in cis. A DEL expression in the father was masked by the presence of a normal *RHD* allele encoded on the partner chromosome. D^{EL} means DEL phenotype and E^{VAR} means altered E antigen expression.

Molecular investigations revealed two other previously unreported alleles. One of these variants, carried by a r″r sample, was characterized by sequencing. A G insertion between Positions 581 and 582 was found to be responsible for a premature stop codon at Position 592 to 594. This allele was dubbed *RHD(581insG)* (Fig. 2B). Adsorption-elution assays failed to detect D antigen expression in this sample (data not shown).

The other genetic structure found in the r′r sample lacking the *RHD*-specific 5′ UTR was analyzed by the BLOODchip microarray platform. The results obtained confirmed a *RHC*/*RHc* heterozygous genotype at the *RHCE* locus. Besides, the formerly described deletion of 11 nucleotides at Position 361 in the *RHD* gene was detected. However, the software failed to establish the *RHD* genotype due to the absence of *RHD* Exon 1– and Exon 2–specific amplification. Further PCR-SSP strategies also failed to detect both *RHD* exons. These findings highly suggest that this allele is a novel hybrid structure that results from homologous recombination between the *RHD(361del11)* null variant and *RHCE* sequences. We named this allele *RHCE(1-2)-RHD($3_{361del11}$-10)* (Fig. 2A). The presence of only one copy of a hybrid *Rhesus box*, together with the phenotypic and genotypic detection of both C and c antigens, suggest that this variant represents a *RHD* allele rather than a *RHCE* variant. RFLP analyses demonstrated the presence of the Intron 1 *RHCE*-specific sequence and the Intron 2 *RHD*-specific sequence indicating that the recombination between *RHD(361del11)* and *RHCE* sequences occurred within the 4.2-kb homologous region surrounding Exon 2 (Fig. 2A).

Overall, molecular analyses showed that the *RHD* deletion is the main cause of the D– phenotype (97.9%) in the population studied (Table 1). However, 2.1% of the samples analyzed carried *DEL* or *null* variants. The presence of *RHD* alleles was most frequently found in D– samples expressing C and/or E antigens (Table 2). Within this latter group, 16.7% were carrying *RHD* alleles, including *null* (11.4%) and *DEL* (5.3%) variants.

DISCUSSION

The determination of the D antigen on RBCs is mandatory in daily transfusion practice to prevent alloimmunization or to avoid hemolytic transfusion reactions in sensitized patients. Comprehensive molecular blood group typing approaches are becoming widespread mainly to facilitate the provision of compatible units in complex scenarios. However, the optimal application of DNA typing requires an exhaustive analysis of the polymorphisms and allele distribution of the blood group genes under study, in particular of the RH system, since a high level of genetic variability was observed in this system among different ethnic groups.[1,2] In a previous study, we analyzed the *RHD* allele pool responsible for altered D antigen expression and determined the frequency of the D– phenotype in the overall Argentinean population, which was 7.2%.[15] In this work we examined the *RHD* locus in routine serologically D– individuals of this admixed population. Even though the *RHD* deletion is responsible for most of the D– phenotype, 2.1% of the samples analyzed carried *DEL* or *null* variants (Table 1). This prevalence is higher than the 0.2% to 0.6% reported in the literature for different regions of Europe.[2-4] More than 50% of the *RHD* alleles found in our study were represented by *RHDψ* and *RHD-CE-D^s* showing the African contribution to the genetic pool of the white population analyzed.[22-24] The high prevalence of both alleles in the D– population studied has led us to implement a genotyping strategy for the noninvasive determination of fetal *RHD* genotype that includes the routine detection of these alleles to avoid false-positive results.

The *RHD-CE-D^s* variant poses a special challenge to blood transfusion because it encodes a complex antigen profile with partial expression of C and e, lack of both hr^B (RH31) and Hr^B (RH34) antigens, and expression of the low-prevalence antigen VS (RH20).[1] In the population

A) Homologous recombination between previously described *RHD* alleles and *RHCE* sequences.

RHD(329T>C)-CE(3-9)-D

RHD-CE(2-9)-D

RHCE-D($3_{361del11}$-10)

B) One nucleotide insertion

RHD(581insG)

Fig. 2. Molecular bases of new alleles. Black boxes indicate *RHD* exons and gray boxes represent *RHCE* exons. Black dots indicate a *RHD* sequence while gray dots indicate a *RHC* sequence. As the origin of Exon 2 in some variants could not be determined because it has an identical nucleotide sequence in *RHD* and *RHC* alleles, it is represented with an empty box. The second allele depicted most likely represent the previously described *RHD-CE(2-9)-D*.

TABLE 2. Distribution of samples carrying *RHD* alleles

	% of *RHD* alleles	
Rh phenotype	In different Rh phenotype groups	In the total D– samples
D–, C/E–	0.7 (8/1200)	0.6 (8/1314)
D–, C/E+	16.7 (19/114)	1.5 (19/1314)

analyzed, this allele was always found in a heterozygous state; thus, patients with the *r^s/dce* genotype may be at risk of anti-C immunization if transfused with r′r units. However, and as a consequence of admixture events, *r^s/dcE* and *r^s/DcE* patients have already been found among samples referred to our laboratory for confirmation of C phenotype due to discrepant results. If unexpected alloantibodies other than anti-C are found in these patients, anti-hr^B (and anti-Hr^B) should be considered as possible specificity(-ies).[23,25] Implementation of molecular detection of the *RHD-CE-D^s* variant would optimize the use of compatible units mainly in patients requiring chronic transfusions.

As shown by others, the occurrence of *RHD* alleles was higher among D– samples expressing C and/or E antigens. However, the frequency of 16.67% found in our D–, C/E+ population (Table 2) is greater than those previously reported for regions of central Europe[4,5] and similar to the prevalence of 13.14% determined in Spain.[26] In fact, our population has a predominantly European genetic component as a consequence of the massive immigration from Spain and Italy at the beginning of the 20th century.

To our surprise, in the group of D–, C/E+ phenotype, we also found a relatively high prevalence (five of 114) of the infrequent *RHD(46T>C)* allele associated with normal or altered E expression.[21] In the five *RHD(46T>C)* samples analyzed in this work, D antigen expression was undoubtedly detected by adsorption-elution assays. However, in some of them a very weak reaction in saline with some IgM anti-D clones was observed. This pattern of reaction makes difficult to classify this variant as a weak D or a DEL phenotype. Considering the negative reaction obtained with the blended anti-D used for the screening of the samples reported in this study, we considered this allele to be responsible for a DEL phenotype. Except for one *RHD(M295I)*, *RHD(46T>C)* was the only *DEL* variant encountered. It is worth mentioning that this is the second observation of a single mutation at Position 16 in the RhD protein. In the DUC-3 variant associated with a DCe haplotype, Trp-16 is changed by Cys—instead of Arg as in *RHD(46T>C)*—but this phenomenon results in an apparently normal D+ phenotype as described in the Rhesus site (http://www.uni-ulm.de/~wflegel/RH/). On the other hand, Residue 16 is involved in C/c polymorphism and has been reported to be associated with weak e antigen expression. Taken together, these observations strongly support the fact that Position 16 plays a crucial role in the antigenicity on both Rh polypeptides. Regarding the Trp16Arg change found in the DEL phenotype, the introduction of a charged residue within the predictive first transmembrane helix of the RhD protein is likely to be disruptive, giving rise to steric hindrance at this position and altering the global fold of the RhD polypeptide. Family studies allowed us to confirm that some *RHD(46T>C)* alleles cosegregated in cis with the also infrequent *RHcE(697C>G, 712A>G, 733C>G, 744T>C)* variant known to be responsible for altered E antigen expression. This rare *RHcE* allele had only been previously reported by Vege and colleagues[27] in one proband with E typing discrepancy.

Interestingly, this is the first observation of the Leu245Val change, mainly involved in altered e antigen expression, associated with aberrant E antigenicity. The description of in cis associations, such as this, may be important for the identification of antibodies to high-frequency Rh antigens facilitating matching efforts for patients with rare or uncommon genotypes.

The finding of *DEL* alleles in our population highlights the importance of genotyping D–, C/E+ donors to properly detect RBC samples with a diminished D antigen expression since it has been demonstrated that DEL RBC units have the potential to cause anti-D alloimmunization in truly D– patients when inadvertently labeled as D–.[7,8] In addition, *DEL* alleles can cause genotype–phenotype discrepancies and should be taken into consideration when performing fetal blood group genotyping.[5,28]

Surprisingly, two different hybrid structures were found in *RHD-CE(3-9)-D* alleles (Fig. 2A). Even though the analysis of intronic polymorphisms does not allow us to determine the *RHD* or *RHC* origin of Exon 2, the finding of the *DVII* characteristic 329T>C mutation, in two of the samples, highly suggests that the breakpoint site in at least some of the *RHD-CE(3-9)-D* variants must lay downstream of the *RHD* Exon 2.

Interestingly, a novel *RHD* frameshift mutation causing a premature stop codon was found. The *RHD(581insG)* allele, characterized by an insertion of G between Nucleotides 581 and 582 in Exon 4 (Fig. 2B), is predicted to encode a 197-amino-acid polypeptide with the first 193 residues identical to the wild-type RhD protein followed by four new frameshift-generated amino acid residues. Taking into account that the T insertion in the *RHD(93insT)* allele generates a premature stop codon at Position 35 and is still responsible for a DEL phenotype[29] we evaluated the sample carrying the *RHD(581insG)* variant by adsorption-elution assays. No D antigen expression was detected, suggesting that this new variant is a *null* allele, which is likely to generate a significantly truncated protein lacking amino acids that are essential for proper integration in the Rh complex and/or epitope expression.

The other novel allele, dubbed in this work *RHCE(1-2)-RHD($3_{361del11}$-10)*, was characterized by the deletion of 11 nucleotides within Positions 361 to 371 and the absence of the *RHD* specific fragment encompassing Exons 1 and 2. The deletion generates a premature stop codon and has been reported to be responsible for a D– phenotype,[26,30] as observed in our sample. Interestingly, the sample's phenotype was C+ and c+, in agreement with the *RHC/RHc* genotype obtained with the BLOODchip (deduced by the detection of the 109-bp insertion in Intron 2, G/C heterozygosity at Position 48, and C/T heterozygosity at Position 307 of the *RHCE* gene). Moreover, the presence of only one copy of a hybrid *Rhesus box* indicated a *RHD* hemizygous status. Although cDNA analysis could not be performed, the data obtained altogether highly suggest that this variant represents a hybrid structure at the *RHD* locus arising from homologous recombination between the previously described *RHD(361del11)* null allele and *RHCE* sequences (Fig. 2A). The breakpoint was localized within a 4.2-kb region, whose long stretch of *RHD* and *RHCE* identical sequence makes it prone to participate in recombination events between these two genes. Worth noting, the *RHD*(*361del11)* was the most frequently null allele found in the Spanish population[26] whose influence in our genetic background has been mentioned previously.

The results obtained in this work enlarge the heterogeneity of the molecular structures that are found at the *RH* locus. The knowledge of the *RHD* allele repertoire in our population is important for the design of *RHD* genotyping strategies and interpretation of the results. Despite the fact that there is not a single approach capable of detecting all *RHD* variants, the prevalence of *RHD null* and *DEL* alleles in the population studied allowed the implementation of reliable *RHD* genotyping approaches for a better management of patients and pregnant women.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

REFERENCES

1. Daniels G. Human blood groups. 3rd ed. Oxford: Wiley Blackwell; 2013.
2. Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. BMC Genet 2001;2: 10.
3. Denomme GA, Wagner FF, Fernandes BJ, et al. Partial D, weak D types, and novel RHD alleles among 33,864 multiethnic patients: implications for anti-D alloimmunization and prevention. Transfusion 2005;45: 1554-60.
4. Polin H, Danzer M, Gaszner W, et al. Identification of RHD alleles with the potential of anti-D immunization among seemingly D– blood donors in Upper Austria. Transfusion 2009;49:676-81.
5. Gassner C, Doescher A, Drnovsek T, et al. Presence of RHD in serologically D-, C/E+ individuals: a European multicenter study. Transfusion 2005;45:527-38.
6. Peng CT, Shih MC, Liu TC, et al. Molecular basis for the RhD negative phenotype in chinese. Int J Mol Med 2003;11: 515-21.
7. Wagner T, Körmöczi GF, Buchta C, et al. Anti-D immunization by DEL red blood cells. Transfusion 2005;45:520-6.
8. Yasuda H, Ohto H, Sakuma S, et al. Secondary anti-D immunization by Del red blood cells. Transfusion 2005;45: 1581-4.

9. Avena S, Via M, Ziv E, et al. Heterogeneity in genetic admixture across different regions of Argentina. PloS One 2012;7:e34695.
10. Catelli ML, Alvarez-Iglesias V, Gómez-Carballa A, et al. The impact of modern migrations on present-day multi-ethnic Argentina as recorded on the mitochondrial DNA genome. BMC Genet 2011;12:77.
11. Cotorruelo C, Biondi C, Borrás SG, et al. Molecular determination of RhD phenotype by DNA typing: clinical applications. Ann Clin Biochem 2000;37:781-9.
12. Wagner FF, Flegel WA. RHD gene deletion occurred in the Rhesus box. Blood 2000;95:3662-8.
13. 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.
14. Gassner C, Schmarda A, Kilga-Nogler S, et al. RHD/CE typing by polymerase chain reaction using sequence-specific primers. Transfusion 1997;37:1020-6.
15. Brajovich ME, Boggione CT, Biondi CS, et al. Comprehensive analysis of RHD alleles in Argentineans with variant D phenotypes. Transfusion 2012;52:389-96.
16. Daniels GL, Faas BH, Green CA, et al. The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. Transfusion 1998;38:951-8.
17. Kemp TJ, Poulter M, Carritt B. A recombination hot spot in the Rh genes revealed by analysis of unrelated donors with the rare D-phenotype. Am J Hum Genet 1996;59:1066-73.
18. Poulter M, Kempt TJ, Carritt B. DNA-based RH typing: simultaneous determination of Rh C and D status using the polymerase chain reaction. Vox Sang 1996;70:164-8.
19. Legler TJ, Maas JH, Kölher 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.
20. Avent N, Martinez A, Flegel WA, et al. The Bloodgen Project of the European Union 2003-2009. Transfus Med Hemother 2009;36:162-7.
21. Ochoa G, Nogues N, Muñiz-Diaz E, et al. Association of altered E RHCE*cE(697G,712G,733G,744C) with altered D RHD*46C. Vox Sang 2012;103(Suppl 1):P-480.
22. Wagner F, Moulds J, Tounkara A, et al. RHD allele distribution in Africans of Mali. BMC Genet 2003;4:14.
23. Pham BN, Peyrard T, Juszczak G, et al. Heterogeneous molecular background of the weak C, VS+, hr B-, Hr B- phenotype in black persons. Transfusion 2009;49:495-504.
24. Touinssi M, Chapel-Fernandes S, Granier T, et al. Molecular analysis of inactive and active RHD alleles in native Congolese cohorts. Transfusion 2009;49:1353-60.
25. Pham BN, Peyrard T, Juszczak G, et al. Analysis of RhCE variants among 806 individuals in France: considerations for transfusion safety, with emphasis on patients with sickle cell disease. Transfusion 2011;51:1249-60.
26. Nogues N, Tarrago M, Subirana L, et al. RHD null alleles in the Spanish population. Vox Sang 2007;93(Suppl 1): P-400.
27. Vege S, Crowley J, Horn T, et al. Diversity of RHCE and identification of twelve new alleles. Transfusion 2010; 50(Suppl 2):S112-040C.
28. Flegel WA. Molecular genetics and clinical applications for RH. Transfus Apher Sci 2011;44:81-91.
29. Flegel WA, von Zabern I, Wagner FF. Six years' experience performing RHD genotyping to confirm D-red blood cell units in Germany for preventing anti-D immunizations. Transfusion 2009;49:465-71.
30. Chen JM, Fichou Y, Jamet D, et al. Small deletions within the RHD coding sequence: a report of two novel mutational events and a survey of the underlying pathophysiologic mechanisms. Transfusion 2013;53:206-10.