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Original Article

RHD and *RHCE* molecular analysis in weak D blood donors and in patients with Rh antibodies against their own corresponding Rh antigen

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Background - The Rh system is the largest and most polymorphic blood group system. The existence of a large number of *RH* alleles results in variant phenotypes that often complicate blood donor phenotyping and the distinction between auto- and allo-antibodies in recipients who have anti-Rh antibodies in the presence of their own corresponding Rh antigen. Knowledge of these variants is necessary in order to make blood transfusion safer.

Materials and methods - Samples from 48 blood donors with serological weak D and from 29 patients who had anti-Rh antibody in the presence of their own corresponding Rh antigen were evaluated molecularly for *RHD* and *RHCE* alleles using a blood-multiplex ligation-dependent probe amplification assay and Sanger sequencing.

Results - Rh variants were found in 45 of the 48 blood donors: 24/45 (53%) were weak D, 2/45 (4%) partial D and 19/45 (42%) were weak and partial D. The remaining three donors (6%) did not show a mutation in the *RHD* allele. Among the 29 patients, 13/29 had anti-e, of whom 4/13 had genotypes that predicted a partial e antigen; 11/29 had anti-D, with 6/11 being identified as partial D; 2/29 had anti-c, of whom 1/2 was predicted to express partial c antigen; 4/29 who had anti-E and 4/29 who had anti-C did not show mutations in *RHCE*C* or *RHCE*E*.

Discussion - It was possible to find individuals with clinically significant Rh phenotypes due to the weak reactivity of the D antigen, detected through serological tests in blood donors. In patients, when found with the anti-Rh antibody in the presence of the same Rh antigen, it is difficult to distinguish an auto-antibody from an allo-antibody by serological tests; in these cases, molecular methods (genotyping) can help us to determine whether there are changes in the *RH* alleles and to discover the nature of the antibody (allo or auto).

Keywords: *Blood donors, blood group antigens, genotype, blood transfusion, autoantibodies.*

INTRODUCTION

The existence of many *RH* alleles results in variant phenotypes that often make blood typing difficult[1]. Currently, more than 650 Rh variants have been reported. Weak D antigen occurs in 0.2 to 1% of Caucasians[2], and can be identified serologically by low reactivity, depending on the anti-D reagent and the method used. In blood donors, these

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variants must be identified as RhD-positive, to avoid alloimmunisation in RhD-negative receptor[3,4].

The Rh system antigens have great ethnic variability, which can be demonstrated by the VS antigen. This is rare in Europeans and Asians but very common in Africans. Alleles associated with the VS antigen can also express partial antigens, resulting in alloimmunisation and formation of clinically significant antibodies that can cause a transfusion reaction, requiring attention, since partial antigens can be undetectable with monoclonal reagents[5-8].

Knowledge of Rh variants in blood donors and patients is necessary to make blood transfusion safer, especially for individuals with sickle cell disease who receive frequent transfusions[9,10]. A compatible transfusion is the best prophylaxis for alloimmunisation in patients, but there is great difficulty in selecting fully compatible red blood cells, especially for patients who produce antibodies against high-frequency antigens or who produce Rh antibodies against their own corresponding Rh antigen[6,11]. Some phenotyping protocols have been developed to reduce the rate of alloimmunisation; however, many patients continue to develop antibodies against the Rh system. In most cases, it cannot be determined whether these unexplained or unexpected antibodies are auto-antibodies or allo-antibodies, and the risks of Rh antibody formation in individuals with altered Rh proteins are not known precisely[12].

Molecular analysis revealed altered *RH* alleles in patients with anti-Rh alloantibodies in the presence of their own corresponding Rh antigen, as well as in blood donors with weak D reactivity[13]. The high prevalence of altered alleles in pre-transfusion tests of patients and blood donors suggests an emerging role for molecular methods, which are effective in detecting and differentiating these alleles. Our aim was to identify *RHD* and *RHCE* variants in blood donors with weak reactivity of the RhD antigen and in patients with antibodies against their own corresponding Rh antigen.

MATERIALS AND METHODS

Study population

A total of 48 blood samples from selected Brazilian donors were collected at a blood bank in São Paulo, after obtaining informed consent.

Additionally, 29 samples from patients who produce Rh antibodies against their own Rh antigen were selected for the study. These patients had distinct diagnoses and came from two hospitals in São Paulo. The data regarding the phenotype and development of allo-antibodies or auto-antibodies were obtained only from the blood bank's electronic files.

Brazil has a multi-ethnic population, particularly in São Paulo, where this study was performed.

Serological studies

D typing of the blood donors and patients was performed with haemagglutination ABO/Rh (2D) gel test cards (Grifols, Parets del Vallès, Spain), using two anti-D reagents: anti-D IgM (clone P3x61) and anti-D IgG + IgM (clones P3X290, P3X35, P3X61, P3X21223 B10). When a reaction of 3+ or weaker was observed with at least one of the two reagents, the blood donor sample was designated as weak D.

The patients' results for the RhCE antigen typing, antibody identification, direct antiglobulin test (DAT), and self-control test were obtained with the haemagglutination technique using gel cards (Grifols, Parets del Vallès, Spain). The eluate test was performed with acid elution using DiaCidel Solution (Bio-Rad/Diamed, Cressier FR, Switzerland). All tests were performed following the manufacturers' instructions.

Molecular analysis of RHD and RHCE

DNA isolation

Genomic DNA was extracted from whole blood, collected into EDTA, using a QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA, USA). The DNA concentration and purity were analysed using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Multiplex ligation-dependent probe amplification assay

RHD and *RHCE* variant alleles and *RHD* zygosity were analysed in all samples from blood donors and patients using a multiplex ligation-dependent probe amplification (MLPA) assay performed according to a previously described protocol[13,14].

DNA sequencing

Sequencing reactions were performed only in samples not fully characterised by the MLPA assay to distinguish and correctly classify the specificity of the *RHD* or *RHCE* alleles present. *RHD* and *RHCE* exon-specific sequencing was done using *RHD*- and *RHCE*-specific primers, as described previously[13,14].

RESULTS

Table I summarises the results obtained with blood donor samples that showed weak serological reactivity of up to 3+ (in a maximum score of 4+), with one or both of the reagents tested. Reactivity discrepancies were also observed between anti-D clones in the same sample.

We found that 45/48 (94%) blood donors had some *RHD* variant allele. A weak D phenotype was predicted in 62% (27/45): *RHD*weak D type 2* (10/28), all of them in a hemizygous state; *RHD*weak D type 38/RHD*01N.01* (7/28); *RHD*weak D type 3* (5/28), one in *trans* to *RHD*DAR1* and four hemizygous; *RHD*weak D type 1/RHD*01N.01* (3/28); *RHD*weak D type 45/RHD*01N.01 (1/28)*; and *RHD*weak partial 15/ RHD*01N.01 (1/28)*.

We also observed that 18/45 (37%) blood donors had a predicted partial D antigen: *RHD*DAR* was found in 16 donors, in three of whom it was associated with *RHD*DIIIa-CE(4-7)-D*, in one each with *RHD*DNB*, *RHD*Ψ*, and *RHD*weak D type 2* and in the other ten with *RHD*01N.01*. The *RHD*DAR* subtypes described in **Table I** were associated in *cis* to *RHCE*ceAR* (11/15) or *RHCE*ce VS*(3/15). The *RHD*DAU5* allele was found in a hemizygous state in two individuals.

In three blood donors, we found the *RHD*01* gene (expressing the RhD antigen) associated with *RHD*01N.01* (an allele that does not encode the RhD antigen), expressing a weak D phenotype.

Regarding *RHCE*, 19/48 (39%) had variant alleles: *RHCE*ceVS* (6/19), *RHCE*ceAR* (11/19), *RHCE*ce-D(9)-c* (1/19) and *RHCE*ce48C* (1/19).

The Rh antibodies detected in the presence of the corresponding Rh antigens in samples from patients were anti-e (n=13), anti-D (n=11), anti-E (n=4), anti-C (n=4) and anti-c (n=2). The DAT and self-control were positive in 22/29 samples and negative in 7/29. Not all antibodies were classified as allo- or auto-antibodies by the primary hospital either because of recent transfusion or because of limited serological techniques available. In this study, we classified the antibodies as allo- or auto-antibodies based on the genotyping results. The results of the DAT and self-control performed on the 29 patients and their respective diagnoses are shown in **Table II**. In the 29 patients' samples subjected to molecular analysis, we found 16 *RHD* and 18 *RHCE* variant alleles.

Anti-RhCE antibodies

Among 13 patients with anti-e, eight exhibited only conventional *RHCE* (**Ce* or **ce*); therefore, the antibodies they carry are classified as auto-antibodies. The other 5/13 patients with anti-e had variants of *RHCE*: *RHCE*ceMO* in *trans* to a conventional *RHCE*cE* (2/5), *RHCE*ceAG* with *RHCE*cE* (1/5), *RHCE*ceVS.03* heterozygous to *RHCE*ceVS.04* (1/5), and *RHCE*ce48C-D(9)-ce* homozygous (1/5). This last patient had the *RHCE* allele in *cis* to *RHD*Ψ* also homozygous and we classified the anti-e as an auto-antibody due to the positive DAT and self-control. Four patients in this group had an allele that encodes a partial e antigen and in all of them it was associated with a hrB-negative and/or hrS-negative phenotype. One patient with *RHCE*ceAG/RHCE*cE* exhibited a concomitant anti-D but had conventional *RHD*01/RHD*01.N.01*.

Of the four patients who had anti-E, three showed only conventional *RHCE* (**ce* and **cE*) and one exhibited a variant allele, *RHCE*ceVS.03* in *trans* to *RHCE*cE*, encoding a partial e antigen, V-negative and hrB-negative phenotype, but the normal E antigen was encoded, and even though the DAT and self-control were negative, we classified this antibody as an auto-antibody (**Table III**).

Two patients had anti-c, and both exhibited *RHCE* variants: *RHCE*ceTI* in *trans* to *RHCE*Ce*, and *RHCE*VS.03* with a conventional *RHCE*cE*, although only the former encodes a partial c antigen and its antibody was classified as an allo-antibody. The other patient with *RHCE*VS.03/ RHCE*cE* had a hybrid allele *RHD*DIIIa-CE(4-7)-D* in *trans* to a conventional *RHD*01*, encoding a partial C. This patient exhibited an anti-c and anti-E, and in this case, too, the DAT and self-control were negative.

Four patients had anti-C, two had only conventional *RHCE* (**Ce* and **ce*) and two had variant *RHCE* alleles: *RHCE*ce48C* in *trans* to a conventional *RHCE*Ce*. Two individuals with conventional alleles also showed anti-e with a concomitant e+ phenotype. All these antibodies were classified as anti-C auto-antibodies.

Anti-D antibodies

Of the 11 patients with anti-D, five had a conventional *RHD*01* allele with *RHD*01N.01*, and six had eight different *RHD* variants: R*HD*DIII.04* with *RHD*DAR1.00* (1/6), *RHD*DAU5* (1/6), *RHD*DAU3* (1/6), *RHD*weak D type 1* (1/6), *RHD*weak D type 33* with *RHD*DAU4* (1/6) and *RHD*DAR3.01* (1/6). It is known that all these variants,

***Table I** - RHD alleles associated with RHCE alleles, the predicted phenotypes and the D reactivity of blood donors*

Alleles		Predicted phenotypes	D Typing Reagents	
RHD	***RHCE***		***Clone 1****	***Clone 2*****
*RHD*Weak D type 45/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Cce	2+	2+
*RHD*DAU5/RHD*01N.01*	*RHCE*ce/RHCE*ce*	Partial D, ccee	2+	2+
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*cE*	Weak D, ccEE	2+	2+
*RHD*Weak D type 38/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	1+	4+
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	1+	1+
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	1+	2+
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	2+	2+
*RHD*DAR1.02/RHD*DIIIa-CE(4-7)-D*	*RHCE*ceVS.02 /RHCE*ceVS.03*	Partial D, C-, E-, partial c, partial e, V+ VS+ hrB-	1+	1+
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	1+	2+
*RHD*DAR1.02/RHD*01N.01*	*RHCE*ceAR/RHCE*Ce*	Partial D, E-, partial c, V+^{w}	2+	2+
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	2+	2+
*RHD*01/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	D+, Ccee	2+	2+
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	2+	2+
*RHD*Weak D type 38/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	1+	1+
*RHD*DAR1.02/RHD*01N.01*	*RHCE*ceAR/RHCE*Ce*	Partial D, E-, partial c, V+^{w}	1+	1+
*RHD*Weak D type 38/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	0	1+
*RHD*Weak D type 38/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	0	1+
*RHD*Weak D type 38/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	0	1+
*RHD*DAR3.01/RHD*01N.01*	*RHCE*ceVS.02/RHCE*Ce*	Partial D, E-, partial c, V+, VS+	2+	1+
*RHD*DAR1.02/RHD*01N.01*	*RHCE*ceAR/RHCE*Ce*	Partial D, E-, partial c, V+^{w}	1+	1+
*RHD*DAR1.02/RHD*DIIIa-CE(4-7)-D*	*RHCE*ceVS.02 /RHCE*ceVS.03*	Partial D, C-, E-, partial c, partial e, V+ VS+ hrB-	1+	1+
*RHD*DAR1.02/RHD*01N.01*	*RHCE*ceAR/RHCE*Ce*	Partial D, E-, partial c, V+^{w}	2+	1+
*RHD*Weak D type 1/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	2+	1+
*RHD*Weak D type 38/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	0	Weak
*RHD*Weak D type 2/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	3+	3+
*RHD*Weak partial 15/RHD*01N.01*	*RHCE*cE/RHCE*ce*	Weak D, ccEe	0	1+
*RHD*DAR1.02/RHD*01N.01*	*RHCE*ceAR/RHCE*ce*	Partial D, ccee, V+^{w}	2+	2+
*RHD*DAR1.02/RHD*01N.01*	*RHCE*ceAR/RHCE*ce*	Partial D, ccee, V+^{w}	2+	2+
*RHD*DAR1.00/RHD*01N.01*	*RHCE*ceAR/RHCE*ce*	Partial D, ccee, V+^{w}	2+	2+
*RHD*DAR1.00/RHD*01N.01*	*RHCE*ceAR/RHCE*ce*	Partial D, ccee, V+^{w}	2+	2+
*RHD*Weak D type 1/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	2+	2+
*RHD*Ψ/RHD*DAR1.00*	*RHCE*ce48C-D(9)-ce/RHCE*ceAR*	Partial D, ccee, V+^{w}	2+	2+
*RHD*Weak D type 3/RHD*01N.01*	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	2+	2+

*Clone 1: anti-D IgM P3x61; **Clone 2: blend of anti-D IgG + IgM P3X290, P3X35, P3X61, P3X21223 B10.

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***Table I** - RHD alleles associated with RHCE alleles, the predicted phenotypes and the D reactivity of blood donors (continued from previous page)*

Alleles		Predicted phenotypes	D Typing Reagents	
RHD	***RHCE***		***Clone 1****	***Clone 2*****
RHD*Weak D type 2/RHD*DAR1	*RHCE*ceVS.02/RHCE*ce*	Partial D, Ccee, V+, VS+	2+	2+
RHD*Weak D type 3/RHD*01N.01	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	2+	2+
RHD*Weak D type 3/RHD*DAR1	*RHCE*ce/RHCE*ceAR*	Weak D, ccee, V+^{w}	2+	1+
RHD*DIIIa-CE(4-7)-D/RHD*DAR3	*RHCE*ceVS.03/RHCE*ceVS.02*	Partial D, E-, partial c, partial e, V+ VS+ hrB-	2+	2+
RHD*Weak D type 3/RHD*01N.01	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	3+	4+
RHD*Weak D type 38/RHD*01N.01	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	0	1+
RHD*Weak D type 2/RHD*01N.01	*RHCE*cE/RHCE*ce*	Weak D, ccEe	1+	2+
RHD*DNB/RHD*DAR1.01	*RHCE*ce/RHCE*ceAR*	Partial D, ccee, V+^{w}	2+	4+
RHD*Weak D type 1/RHD*01N.01	*RHCE*ceVS.10/RHCE*ceVS.01*	Weak D, E-, partial c, partial e, V+, VS+, hrB+^{w}	1+	1+
RHD*01/RHD*01N.01	*RHCE*Ce/RHCE*ce48C*	D+, Ccee	0+	1+
RHD*01/RHD*01N.01	*RHCE*Ce/RHCE*ce*	D+, Ccee	0+	2+
RHD*Weak D type 3/RHD*01N.01	*RHCE*Ce/RHCE*ce*	Weak D, Ccee	3+	3+
RHD*Weak D type 2/RHD*01N.01	*RHCE*cE/RHCE*ce*	Weak D, ccEe	0	2+
RHD*DAU5/RHD*01N.01	*RHCE*ce/RHCE*ce*	Partial D, ccee	2+	2+
RHD*DAR1.00/RHD*01N.01	*RHCE*ceAR/RHCE*ce48C*	Partial D, ccee, V+^{w}	2+	2+

*Clone 1: anti-D IgM P3x61; **Clone 2: blend of anti-D IgG + IgM P3X290, P3X35, P3X61, P3X21223 B10.

with the exception of the *RHD* weak D type 1*, are capable of causing alloimmunisation, explaining the development of anti-D alloantibodies in these patients.

Altered *RHCE* alleles were also detected in most of the patients with anti-D and a D-positive phenotype, all in *cis* to the altered *RHD*. The *RHCE* variant alleles in decreasing order of frequency were: *RHCE*ce48C* (n=3), *RHCE*ceVS*.02 (n=2), and *RHCE*ceAR* (n=1). Of the 11 patients with anti-D, five had their antibodies defined as auto-antibodies because of a positive DAT, a positive self-control and absence of a *RHD* mutation.

DISCUSSION

The interpretation of D typing in blood donors using serological methods can be complex, because some variants of the D antigen with low antigenic density can be undetectable by methodologies with low sensitivity. Consequently, blood components can be mislabelled as RhD-negative, exposing RhD-negative patients to the risk of anti-D alloimmunisation.

In serological investigations, the main difficulties occur in patients with sickle cell disease who are of African descent and with great miscegenation, which makes the presence of non-detectable RhD and RhCE variants common. The same occurs in patients with oncological diseases and other haematological diseases who receive periodic transfusions because of chemotherapy. In general, the *RH* variant is suspected only after alloimmunisation.

In this study, among the blood donor population, we found weak D reactivity in 48 samples, in one or both clones tested, and reactivity discrepancy between the two clones in the same sample, in 45 of the 48 cases in association with *RHD* mutations. Routine serological techniques are not able to differentiate between weak D and partial D, but they detect the weak expression of the D antigen, suggesting the presence of *RHD* and *RHCE* variant alleles. Blood donors with this condition should be studied molecularly to facilitate the process of finding red blood cells for recipients in need of the same mutations.

RhD serological discrepancies are normally associated with ethnicity. Studies conducted in Europe analysed the frequency of *RHD* alleles and found that 95% of

***Table II** - Results of the self-control and direct antiglobulin test, and diagnosis of the patients with anti-Rh antibodies with the corresponding antigen*

ID	Self-control	DAT	Diagnosis
P1	Positive	Positive	Lupus + haemophilia
P2	Positive	Positive	Sickle cell disease
P3	Positive	Positive	Multiple myeloma
P4	Positive	Positive	Cancer
P5	Positive	Positive	Multiple myeloma + AIHA
P6	Negative	Negative	Gestation
P7	Positive	Positive	Sickle cell disease
P8	Negative	Negative	Sickle cell disease
P9	Positive	Positive	Sickle cell disease
P10	Positive	Positive	Cancer
P11	Positive	Positive	Sickle cell disease
P12	Positive	Positive	Haemophilia + AIDS
P13	Positive	Positive	Heart disease
P14	Positive	Positive	Heart disease
P15	Positive	Positive	Sickle cell disease
P16	Positive	Positive	Sickle cell disease
P17	Positive	Positive	AIHA
P18	Positive	Positive	Sickle cell disease
P19	Positive	Positive	Cancer
P20	Negative	Negative	Cancer
P21	Negative	Negative	Diabetes
P22	Positive	Positive	Kidney disease
P23	Positive	Positive	Kidney disease
P24	Negative	Negative	Cancer
P25	Positive	Positive	ITP
P26	Negative	Negative	Cancer
P27	Negative	Negative	Sickle cell disease
P28	Positive	Positive	Acute myeloid leukemia
P29	Positive	Positive	Sickle cell disease

AIDS: acquired immunodeficiency syndrome; AIHA: autoimmune haemolytic anemia; ITP: idiopathic thrombocytopenic purpura.

Caucasian individuals with weak D antigen expression are *RHD*weak D type 1* to *3*[15]. In other studies, in the Brazilian population, *RHD*weak D type 1* was the most frequently found variant[16,17], in contrast to our results, in which this variant was the least found.

Through gene sequencing, we detected seven (14%) individuals with the 833G>A mutation in exon 6 of *RHD*. This mutation expresses the weak D antigen, with an antigenic density of 60 to 80 sites per cell, characterising *RHD*weak D type 38*, which is very common in the Portuguese population and is found in 1.5% of Brazilian Caucasians with the Ccee phenotype[18]. In the current study, all samples characterised as *RHD*weak D type 38* were associated with the *RHCE*Ce* allele. It has been reported that some anti-D clones are not able to detect mutations that lead to low antigenic density, such as *RHD*weak D type 38* or *RHD*weak partial 11*[1]; in this case, a sensitive methodology was able to detect one of these variants.

In approximately 35% of donors, we observed the *RHD*DAR* allele, a variant that characterises a partial D antigen and is almost always in *cis* in the *RHCE*ceAR* variant[19]. In our current study, we found 12 (25%) donors with the concomitant presence of these two alleles, as previously observed in another study in the same population[20]. The *RHD*DAR* allele is a predominant phenotype in Africans and the frequency found in the Brazilian population confirms the high degree of African ancestry[17,19]. The *RHCE*ceAR* variant encodes the c partial and/or e partial antigens, the low-frequency antigen V and does not express the high-frequency antigen hrS. The other 5/17 blood donors with the *RHD*DAR* allele had the *RHCE*ceVS* variant in *cis*, which expresses the low-frequency antigen VS and does not encode the high-frequency antigen hrB. Patients of African descent may develop complex allo-antibodies, such as anti-hrB and anti-hrS, making blood transfusion compatibility a challenge. Based on the initial identification of *RHD*DAR* in blood donors, *RHCE* mutations can be easily detected and make the red blood cell selection process safer for patients with a clinically significant *RH* genotype.

The wild-type hemizygous *RHD* gene, found in 6% of donors, had reactivity from 1+ to 2+ (on a scale from 0 to 4+). In all cases it was associated with the *RHCE*Ce* gene, two heterozygous to *RHCE*ce*, and one to *RHCE*ce48C*.

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***Table III** - Results of the serological and molecular studies in the group of patients (n=29)*

ID	Alleles		Predicted phenotypes	Probable interpretation	
	RHD	***RHCE***		**Allo-antibody**	**Auto-antibody**
P1	*RHD*DIII.4/RHD*DAR1.00*	*RHCE*ceVS.02/RHCE*ceAR*	Partial D, C-, E-, partial c and e, VS+, V+w, hrB- and hrS-	Anti-D	
P2	*RHD*01/RHD*01N.01*	*RHCE*ceTI/RHCE*Ce*	D+, E-, partial c	Anti-c	
P3	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*ce*	D+, ccee		Anti-D
P4	*RHD*01/RHD*01*	*RHCE*Ce/RHCE*Ce*	D+ CCee		Anti-C + e
P5	*RHD*01/RHD*01N.01*	*RHCE*ceAG/RHCE*cE*	D+, C-, partial e, hrB-, CEAG-	Anti-e	Anti-D
P6	*RHD*DAU5/RHCE*01N.01*	*RHCE*ce/RHCE*ce48C*	Partial D, ccee	Anti-D	
P7	*RHD*01/RHD*01*	*RHCE*ce/RHCE*Ce*	D+, Ccee		Anti-e + C
P8	*RHD*01/RHD*DAU0*	*RHCE*cE/RHCE*ceMO*	D+, C-, partial e, hrB-, hrS-, CEVF-	Anti-e	
P9	*RHD*Ψ/RHD*DIIIc*	*RHCE*ce/RHCE*Ce*	Partial D, Ccee		Anti- e
P10	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*cE*	D+, ccEe		Anti-e
P11	*RHD*DAU3/RHD*01N.01*	*RHCE*ce48C/RHCE*ce*	Partial D, ccee	Anti-D	
P12	*RHD*01/RHD*01N.01*	*RHCE*ce48C/RHCE*Ce*	D+, Ccee		Anti-C
P13	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*Ce*	D+, Ccee		Anti-D
P14	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*cE*	D+, ccEe		Anti-e
P15	*RHD*01/RHD*01N.01*	*RHCE*cE/RHCE*ceVS.01*	D+, C-, partial e, hrB+w/-, V+, VS+		Anti-D
P16	*RHD*Ψ/RHD*Ψ*	*RHCE*ce48C-D(9)-ce/RHCE*ce48C-D(9)-ce*	D-, ccee		Anti-e
P17	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*cE*	D+, ccEe		Anti-E
P18	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*cE*	D+, ccEe		Anti-E + e
P19	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*cE*	D+, ccEe		Anti-E
P20	*RHD*Weak D type 1/RHD*01N.01*	*RHCE*ce/RHCE*Ce*	Weak D, Ccee	Anti-D (Immunoglobulin)	
P21	*RHD*DIIIa-CE(4-7)-D/RHD*01*	*RHCE*ceVS.03/RHCE*cE*	Partial D, C-, partial e, V-, VS+, hrB-, hrS-		Anti-E+c
P22	*RHD*01N.01/RHD*01N.01*	*RHCE*ce/RHCE*ce*	D-, ccee		Anti-e
P23	*RHD*01/RHD*01N.01*	*RHCE*ce/RHCE*Ce*	D+, Ccee		Anti-D
P24	*RHD*Weak D type 33/RHD*DAU4*	*RHCE*ce/RHCE*ce48C*	Partial D, ccee	Anti-D	
P25	*RHD*01/RHD*01*	*RHCE*Ce/RHCE*Ce*	D+, CCee		Anti-e
P26	*RHD*DAR3.01/RHD*01N.01*	*RHCE*cE/RHCE*ceVS.02*	Partial D, C-, partial e, V+, VS+, hrB-	Anti-D	
P27	*RHD*01/RHD*DAU0*	*RHCE*cE/RHCE*ceMO*	D+, C-, partial e, hrS-, hrB-, CEVF-	Anti-e	
P28	*RHD*01/RHD*01N.01*	*RHCE*ce48C/RHCE*Ce*	D+, Ccee		Anti-C
P29	*RHD*DIIIa/RHD*01*	*RHCE*ce.VS.03/RHCE*ce.VS.04*	D+, C-, E-, partial e, VS+, V+, hrB-	Anti-e	

When the *RHD* gene is associated in *trans* with the dCe haplotype, there is a possible decrease in RhD protein expression[21]. Moreover, intronic polymorphisms may be present, which are not detected in the sequencing of *RHD* exons and which could affect the expression of RhD proteins.

RBC alloimmunisation can occur in a variety of clinical conditions. Twenty-nine patients who produce Rh antibodies against their own corresponding Rh antigen were found in this study; however, determining the nature of such antibodies (auto- or allo-antibodies) is a challenge for haemotherapy services due either to recent transfusion or to limited serological methods available. Genotyping can solve these cases. After molecular analysis of the *RHD* and *RHCE* genes, it was observed that, in five cases in which antibodies could be serologically interpreted as probable auto-antibodies, the molecular investigation showed the presence of partial antigens with the possible production of partial allo-antibodies.

We observed alloanti-D in the presence of a partial D antigen in 17% of the population of patients. We found three patients with alloanti-D who were positive for *RHCE* and *RHD* variants, concomitantly. These patients are, therefore, susceptible to alloimmunisation against partial RhCE antigens in addition to partial D, as already observed in previous studies in which individuals with *RHD* variants also have mutations in *RHCE*[20].

One individual (P20) did not show a positive self-control test and DAT and was identified as having *RHD*weak D type 1*; after medical analysis, we found that this was due to the administration of anti-D immunoglobulin during pregnancy in another hospital, 3 months before the tests. RhD immunoglobulin can persist and be detected in a pregnant woman's serum beyond the expected limit of 6 weeks[22]. Another patient (P21) showed a negative DAT and self-control test, without mutations in *RHCE* alleles; despite the patient presenting with anti-E and anti-c in the presence of E and c antigens, we classified this as an auto-antibody. However, further molecular tests should be performed to confirm the presence or absence of variants in the patient. We should also consider the possibility that the patient has produced an antibody against low-incidence Rh antigens present in African-Brazilian blood donors, as demonstrated in other studies with African-American donors[12,13]. In rare cases, the self-control test can be negative even if there is an auto-antibody because the antigen receptor expression is weaker than that of the red blood cells used in the antibody identification panel. The DAT can also be negative, even in the presence of auto-antibody since the number of IgG molecules is less than the test used can detect[23].

Misclassification of anti-D antibodies as allo-antibodies instead of auto-antibodies can lead to the selection of RhD-negative blood for transfusion, so patients of African descent with sickle cell disease can be exposed to alloimmunisation to antigens of other blood group systems, such as Fya, Jkb or S, phenotypes common in the RhD-negative Caucasian population[11]. There are increasing reports of patients with sickle cell disease who develop Rh antibodies against their own Rh antigens[12]. Auto-antibodies may not be as relevant as allo-antibodies and the decision to respect them or not in the clinical evaluation of the patient, and the provision of a compatible blood phenotype with a negative antigen for the autoantibody are complex. It is, therefore, important to distinguish between auto- and allo-antibodies in order to improve the management of blood components, prevent the risk of transfusion reactions, avoid wasting phenotypically compatible red blood cells and avoid alloimmunisation[11,24].

The development of auto-antibodies with Rh specificity in patients without the aberrant *RHD* and *RHCE* genes may be related to diseases such as systemic lupus erythrematosus and other disorders of the immune system. In addition, some antibodies can be induced by drugs such as methyldopa[25].

RH genotyping can guide transfusion conduct in cases of unexplained antibodies that we do not know how to differentiate between auto- or allo-antibodies and help find compatible blood donors to try to provide *RH-* genotyped matched transfusions, particularly for sickle cell disease patients. Chou *et al.*[26] demonstrated that *RH* genotype matching for transfusion support in patients with sickle cell disease would be possible. Nevertheless, this requires enough blood donors, and the cost of genotyping is high.

CONCLUSIONS

In summary, this study demonstrates the challenges in the routine determination of whether an anti-Rh antibody

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is an auto-antibody or an allo-antibody and shows us that the available serological methods are limited and cannot help in some cases. However, serological testing can be a way to find blood donors to match patients with clinically relevant altered *RH* genotypes and facilitate blood screening. A limitation of this study is that the data do not provide the patient's entire transfusion history. Although further research is needed to define the immunogenicity and clinical relevance of antibodies associated with a partial antigen, we showed that *RHD* and *RHCE* genotyping in blood donors with weak reactivity and molecular research of patients with Rh antibodies against their own corresponding Rh antigen are useful to develop transfusion protocols and manage a red blood cell inventory.

AUTHORSHIP CONTRIBUTIONS

TCSS is the main author. TCSS, BRC, SSMC, and JOB conceived and designed the study, analysed and interpreted the data, wrote the manuscript and approved the final version. MMOB and DML provided study material. TCSS, BRC and AKC performed laboratory testing. TCSS and BRC collected, assembled, analysed and interpreted the data.

The Authors declare that they have no conflicts of interest.

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