Comprehensive phenotypic and molecular investigation of RhD and RhCE variants in Moroccan blood donors

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Background. To date, more than 650 (weak and partial) Rh variants have been reported. Nature and frequency of these variants are known to be ethnodependent. In transfusion medicine, their identification is important to ensure blood safety. The aim of this study is to investigate and describe the nature and estimate the frequency of Rh variants in blood donors in Morocco by serological tests and molecular analysis.

Materials and methods. Blood samples from 4,458 blood donors were collected and typed for Rh antigens (D, C, c, E and e) by an automated system with monoclonal antibodies. RhD-negative samples were tested for weak D expression by indirect antiglobulin test (IAT), as well as weak C, c, E, and e expression with monoclonal antibodies, by column agglutination technique. All samples exhibiting a weak D agglutination by the automated system and IAT were tested for partial D. *RHD* and *RHCE* genes were analysed by quantitative multiplex PCR of short fluorescent fragments (QMPSF) and/or Sanger sequencing.

Results. 4,038 (90.58%) and 420 (9.42%) samples were respectively typed serologically as D-positive and D-negative, including 23 (0.52%) presenting with a weak D phenotype. In 21 weak D samples investigated by molecular analysis, *RHD*weak D type 4.0* was found to be the most prevalent variant allele (n=11), and a novel *RHD*(V270A) missense allele was found once. Variant Rh CcEe antigen expression was observed in 17 samples carrying 20 variant *RHCE* alleles, including a novel *RHCE*ce(499G)* missense allele (p.M167V).

Discussion. For the first time, molecular genetics of the Rh system was investigated in the Moroccan population. On the basis of our data and in order to optimise donor/recipient matching to prevent from a potential risk of alloimmunisation in recipients, we suggest that 1) quality control of serological reagents and screening strategies must be reviewed in Morocco, and 2) molecular analysis should be implemented and performed in blood donor centers.

Keywords: Rh phenotype, blood transfusion, *RHD* variants, *RHCE* variants.

Introduction

The Rhesus (Rh) blood group system is a complex, polymorphic system including at least 55 antigens[1], of which the most clinically relevant are D, C, E, c, and e[2]. RhD antigen incompatibility is the principal cause of haemolytic disease of the newborn and haemolytic transfusion reactions[3]. The D antigen is a mosaic comprising at least 30 different epitopes defined by human monoclonal antibodies[4]. Individuals with red blood cells lacking one or more epitopes, i.e. partial D, can produce alloanti-D directed against the missing D epitope(s), while a weak D phenotype consists of a reduced number of D antigen sites at the red blood cell membrane with no alteration of epitope expression[5]. This phenotypic variability is also encountered in the C, c, E, and e antigens[5]. Extensive molecular analysis of samples with weak and/or partial phenotypes in various populations has revealed that the molecular heterogeneity by far surpasses the serological heterogeneity, and more than 650 variant *RH* alleles, with substantial ethnic specificity resulting in variant phenotype expression, have been identified so far[6,7].

Detection of Rh variant phenotypes depends directly on various factors, such as routine serological

techniques and reagents[8-10]. Discrepancies in typing may lead to serological omission of these variants resulting in incompatible transfusions that could have clinical consequences, ranging from transfusion inefficiency to the patient's death[11,12]. Some Rh variant samples are not detected by serological techniques and can only be distinguished by molecular studies. Molecular characterisation thus prevents alloimmunisation and facilitates management of transfusion therapy and rational Rh immunoprophylaxis by administration of human anti-D immunoglobulin in D-negative pregnant women[10,13].

In Morocco, the prevalence of weak D antigen in samples routinely typed as D-negative has been reported to be ~0.4% in the general population, and up to 15% in D-negative, C/E+ samples[8,14]. In a recent study conducted at the Regional Blood Transfusion Centre of Rabat in Morocco, ~50% of cases of alloimmunisation towards the antigens of the Rh system were shown to be associated with expression of variant D antigens, while ~24% of cases were related to CcEe antigens[15]. These results suggest that molecular typing is required in Morocco to reduce the risk of alloimmunisation, to enable better management of blood units and, ultimately, to improve transfusion outcomes. As the molecular basis of Rh variant expression is unknown in the Moroccan population, we thought to investigate and describe the nature and frequency of these variants at both phenotypic and molecular levels in blood donors.

Materials and methods

Blood samples and Rh serology testing

The study and consent protocols were approved by the Medical Ethics Committee, Faculty of Medicine and Pharmacy of Casablanca. Blood samples from 4,458 blood donors were collected into tubes containing EDTA at the Regional Blood Transfusion Centre of Casablanca (CRTSC). Rh antigens (D, C, c, E, and e) were tested routinely by an electromagnetic technology with a DuoLys microplate (Diagast, Loos, France) in a fully automated Qwalys® 3 system (Diagast). Tests were performed with monoclonal anti-D (clone P3x61), anti-C (P3x25513G8 + MS24), anti-c (951), anti-E (906), and anti-e (P3GD512 + MS63) antibodies (Diagast). Samples showing a weak D antigen agglutination graded as ≤2+ with the Qwalys® 3 automated system were considered as suspected weak D. In parallel, Rh D-negative samples were tested for weak D expression in the antihuman globulin phase with monoclonal anti-D antibodies (clones P3x61 + P3x21223B10 + P3x290 + P3x35, Diagast), as well as weak C, c, E, and e expression with the aforementioned clones, by a column agglutination technique. Suspected and confirmed weak D samples were then tested for partial D with a panel of nine monoclonal IgM and IgG anti-D antibodies (D-Screen Kit, Diagast) using the Coombs Anti-Human IgG Card system according to the manufacturer's instructions (Bio-Rad, Gladesville, NSW, Australia). These reagents were selected for their ability to define specific reaction profiles of the most frequent partial D variants (i.e. DII, DIIIa, DIIIb, DIIIc, DIVa, DIVb, DVa, DVI, DVII, DFR, DBT, DHAR, and DHMi). Both Rh control and Coombs control cells were used to ensure a highly diagnostic sensitivity and specificity, regarding RhD detection.

Genomic DNA extraction and molecular typing

Genomic DNA was isolated from EDTA-anticoagulated blood samples by the QIAamp DNA Blood Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions and quantified by optical density measurement with Nanodrop 1000 (Nanodrop Technologies, Wilmington, DE, USA).

RHD and *RHCE* genes were first analysed by quantitative multiplex polymerase chain reaction of short fluorescent fragments (QMPSF) for assessing potential exon copy number variations and identifying hybrid *D-CE* alleles, and then by Sanger sequencing. QMPSF and sequencing conditions were as described elsewhere[16-18]. Output sequencing data were analysed with Sequencher version 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA) and Alamut-visual version 2.9.0 (Interactive Biosoftware, Rouen, France).

Results

Rh phenotype

In the 4,458 Moroccan blood donors, 4,038 (90.58%) and 420 (9.42%) samples were respectively typed as D-positive (D+), including 19 presenting weak D agglutination (≤2+), and D-negative (D–) by routine automated serological analysis. Further

Table I - Rh phenotype in Moroccan blood donors.

Rh phenotype	Occurrence	Frequency (%)
DCcee	1,724	38.67
Dccee	860	19.29
DCCee	679	15.23
DccEe	371	8.32
DCcEe	363	8.14
DccEE	35	0.79
DCCEe	6	0.13
dccee	386	8.66
dCcee	25	0.56
dccEe	8	0.18
dCcEE	1	0.02
Total	4,458	100.00

manual testing with monoclonal anti-D revealed four additional weak D samples among the 420 D-negative samples, which had not been detected routinely by Qwalys (0.95%, 4/420). Taken together, our results show that the prevalence of weak D in Moroccan blood donors is 0.52% (23/4,458).

The Rh CcEe phenotype was determined concomitantly in the same samples (Table I). Automated screening revealed 12 samples with weak antigen intensity (e: n=7; E and e: n=2; C: n=1; C and e: n= 1; c and e: n=1). Five additional samples were found to be weak e (n=4) or weak E and e (n=) by manual testing in the 420 D– samples.

Overall, a total of 38 samples (weak D: n=21; weak D and e: n=2; weak C/c/E/e: n=15) were selected for subsequent molecular analysis of the *RHD* and/or *RHCE* gene(s).

*RHD*weak D type 4.0 allele* is the most common *RH* gene variant in Moroccan blood donors

As DNA could not be extracted from two samples because of limited sample volumes, the *RHD* gene was further investigated in 21/23 weak D samples by QMPSF and Sanger sequencing. *RHD*weak D type 4.0*[19] was shown to be the most common variant *RHD* allele in the Moroccan blood donors (11/21), while other variant alleles were found once at the hemizygous state (Table II). The molecular basis of the weak D phenotype could not be elucidated in three samples carrying an apparent wild-type *RHD* allele in *trans* with either a hybrid *D-CE(7)-D* allele, or another apparent wild-type *RHD* allele, or a novel single-nucleotide missense allele, i.e. *RHD*(c.809T>C), resulting in the substitution of a valine amino acid by an alanine residue at position 270 of the RhD polypeptide (p.V270A) (Figure 1A, Table II).

Table II - *RHD* variants in Moroccan weak D blood donors.

***RHD* allele**	**Occurrence**	**Rh CcEe phenotype**
Weak D type 4.0[a]	10	C–c+E–e+
Weak D type 4.0[a]	1	C+c+E–e+
Weak D type 1[a,b]	1	C+c+E–e+
Weak D type 2[a,b]	1	C–c+E+e+
Weak D type 3[a]	1	C+c+E–e+
Weak D type 61[a]	1	C+c+E–e+
DAU-0[a]	1	C-c+E–e+
DOL1[a]	1	C–c+E–e+
DVII[a]	1	C+c+E–e+
RHD/RHD-CE(7)-D	1	C–c+E+e+
RHD/RHD	1	C+c+E–e+
RHD/RHD(c.809C)	1	C+c+E–e+
Not tested	2	C+c+E–e+
Total	23	-

[a]Hemizygous; [b]identified by gel card testing; *RHD*: conventional wild-type *RHD* allele. Weak D phenotype samples included 19 suspected weak-D presenting automated agglutination graded as ≤2+ and four others detected by manual indirect Coombs testing. *RHD* variants were determined by a molecular sequencing technique.

D-epitope profile was assessed with a commercial kit in the 23 samples of interest (Table III). A full D-epitope profile was observed in 16/23 weak D samples. A partially reactive D-epitope profile, i.e. absence of epitope 8.2, was found in five samples, including those carrying the *RHD*weak D type 1*,

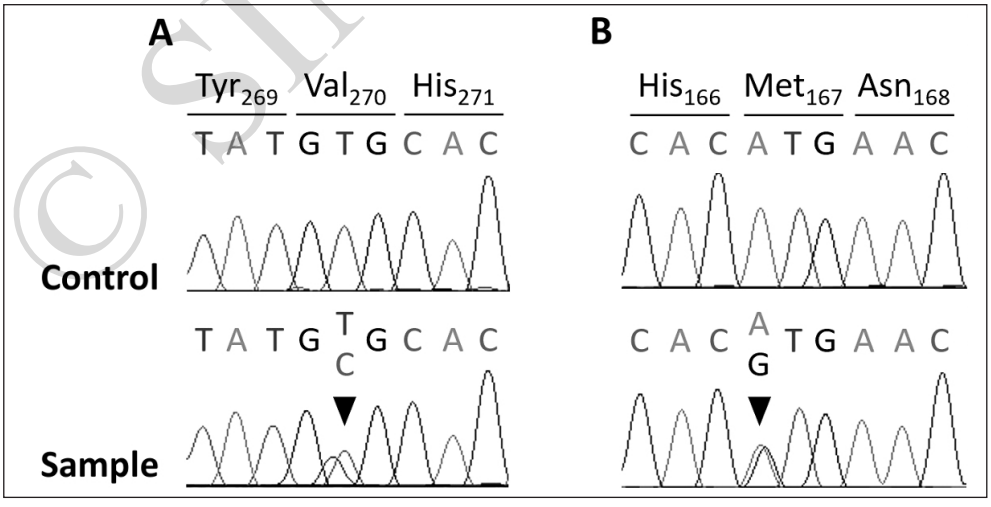

Figure 1 - Novel c.809T>C (p.Val270Ala) (A) and c.499A>G (p.Met167Val) (B) missense variations in the *RHD* and *RHCE* genes, respectively, in Moroccan blood donors.
Control: wild-type *RHD* (L08429) and *RHCE* (cc) (DQ322275) sequences; arrowhead indicates the nucleotide changes; amino acids with their respective positions in the proteins are indicated above the nucleotide sequences.

Table III - Epitope profile of RhD variant samples in Moroccan blood donors.

			Weak D type 1	Weak D type 2	Weak D type 3	Weak D type 4.0	Weak D type 61	*DAU-0*	*DOL1*	*DVII*	*RHD/D-CE(7)-D*	*RHD/RHD(c.809C)*	*RHD/RHD*	ND
Occurrence			1	1	1	11	1	1	1	1	1	1	1	2
Clone	*Ig class*	*Epitope*[a]												
HM10	IgM	6.6	+	+	+	+	+	+	+	+	+	+	+	+
HM16	IgG	6.4	+	+	+	+	+	+	+	+	+	+	+	+
P3x61	IgM	6.1	+	+	+	+	+	+	+	+	+	+	+	+
P3x35	IgG	5.4	+	+	+	+	+	+	+	+	+	+	+	+
P3x21211F1	IgM	8.2	–	–	–	+	+	+	–	–	+	+	+	–
P3x21223B10	IgM	9.1	+	+	+	+	+	+	+	+	+	+	+	–
P3x241	IgG	5.4	+	+	+	+	+	+	+	+	+	+	+	+
P3x249	IgG	2.1	+	+	+	+	+	+	+	+	+	+	+	+
P3x290	IgG	3.1	+	+	+	+	+	+	+	+	+	+	+	+

[a]D epitope nomenclature in accordance with Scott[37]. ND: not determined; IgM: immunoglobulin class M; IgG: immunoglobulin class G.

*RHD*weak D type 2*, *RHD*weak D type 3*, *RHD*DOL1*, and *RHD*DVII* alleles, which is concordant with the findings of previous studies[20-22]. The two remaining samples that could not be genotyped showed absence of epitopes 8.2 and 9.1.

In the 17 samples that underwent *RHCE* genotyping, the most frequent allelic background was found to be *RHCE*ce* (n=27), including 20 variant alleles (74%) with a typical African identity: *RHCE*ce.01* (n=4), *RHCE*ceAG* (n=4), *RHCE*ceMO* (n=3), *RHCE*ce(48C)-D(9)-ce* (n=3) known to be *cis*-associated with *RHD08N.01* (i.e. *RHDΨ*), *RHCE*ceVS.01* (n=2), *RHCE*ceVS.05* (n=1), *RHCE*ce.30* (n=1), *RHCE*ce(48C, 105T, 733G, 744C, 1025T)* (n=1), and the novel *RHCE*ce(499G)* allele (Figure 1B). This aforementioned missense (c.499A>G, p.M167V) allele, which was found in *trans* with *RHCE*ce(48C)-D(9)-ce* (Table IV), is thought to be *cis*-associated with *RHD* gene deletion based on QMPSF data (*data not shown*). The other allelic backgrounds were *RHCE*Ce* (n=4), including one variant *RHCE*CeRN.01* allele, followed by the conventional *RHCE*cE* (n=2) and *RHCE*CE* (n=1) alleles (Table IV).

Table IV - *RHCE* variants in weak CcEe Moroccan blood donors.

Most likely *RHCE* genotype		Occurrence	Serological test
Allele 1	***Allele 2***	***n=17***	
ce	ceVS.01	2	Weak e
ce	ceAG	2[a]	Weak e
ce	ce.01	2[a]	Weak e
Ce	ceVS.05	1	Weak e
Ce	ce.30	1	Weak C
cE	CeRN.01	1	Weak e
cE	ceMO	1	Weak e
ceMO	ceMO	1	Weak c/e
ce	ce(48C)-D(9)-ce	1	Weak E/e
Ce	ce(48C)-D(9)-ce	1	Weak E/e
ce(48C)-D(9)-ce	ce(499G)	1[a]	Weak E/e
ce.01	ceAG	1	Weak e
ce.01	CE	1	Weak C/e
ceAG	ce(48C, 105T, 733G, 744C, 1025T)	1	Weak e

[a]Identified by gel card testing. *ceAG*: *ce.06*; *ceMO*: *ce.07.01*; *ceVS.01*: *ce.20.01*; *ceVS.05*: *ce.20.05*; *CeRN.01*: *Ce.10.01* (New York Blood Center RHCE database)[7].

Discussion

In a total of 23 weak D samples detected in blood donors (23/4,458; 0.52%), *RHD*weak D type 4.0* was found to be the most prevalent weak *D* allele (11/21, 52%). This allele was previously shown to be the most prevalent variant *RHD* allele in Tunisia, with a frequency of 2.1% in D+ samples[23]. All samples genotyped as weak *D* type 4.0 showed a complete D epitope profile, as reported in other studies[20,24]. While a case of alloanti-D has been reported in a patient[25], recent recommendations suggest that *RHD weak D type 4.0*-genotyped patients may be transfused with D+ red blood cells[26,27]. In Morocco there are no data about adverse clinical effects in weak D type 4.0 recipients as follow up of recipients to monitor risks and complications is not conducted. Routinely, the anti-D reagent used in our transfusion centre allows detection of weak D type 4.0, so such a

carrier would be typed as D+, and then would either be managed as and transfused with D+ red blood cell units, or would not receive anti-D immunoglobulin in the case of pregnancy. However, caution should be taken in extrapolating serological results, relying on available antisera reagents, without considering clinical evidence. At this stage, no decision on weak D type 4.0 can be taken until more data are available.

*RHD*weak D type 1*, *RHD*weak D type 2* and *RHD*weak D type 3* alleles are rare in Morocco (1/4,458, 0.02% each). As weak D type 1, 2 and 3 individuals are not at risk of developing anti-D when exposed to D+ red blood cells, pregnant women and patients with these weak D phenotypes can be managed safely as D+[28]. It should, however, be noted that mistyping these variants as D– by serology may have clinical consequences in recipients. In this work, individuals carrying weak *D* type 1 or weak *D* type 2 allele were missed by routine serological techniques, suggesting importantly that the strategy used in Moroccan Blood Transfusion Centres should be adapted and improved to identify these variants. Our genotyping strategy also revealed a novel missense *RHD* allele (c.809T>C, p.V270A), at the same nucleotide position defining the weak *D* type 1 allele (c.809T>G, p.V270G)[19]. Although a complete D-epitope profile was observed with our range of antibodies in this novel variant (Table III), additional data will be required to assess the clinical significance of this amino acid change.

As expected, no partial DVI phenotype was found. The monoclonal anti-D reagents used routinely in the Blood Transfusion Centre of Casablanca enable the detection of this phenotype. However, this strategy can increase the risk of anti-D alloimmunisation for blood donors as recipients or females of childbearing potential with a partial DVI phenotype, therefore caution is needed when adopting the following strategy[29,30].

A total of 17 samples with weak C/c and/or E/e phenotypes were detected in 4,458 blood donors (0.38%). In these samples, additionally to the four common *RHCE* alleles (i.e. *ce*, *Ce*, *cE*, and *CE*), we found ten variant alleles which are known to alter quantitatively and/or qualitatively the expression of either antigen and to be clinically relevant[23,31-38]. Although few samples were genotyped in our study, this observation seems to reveal the large polymorphism of the *RHCE* gene in the Moroccan population, as already reported in various populations of African descent[2]. These preliminary results highlight the need to carry out a larger molecular epidemiology study to determine the frequency and distribution of variant *RHCE* alleles in the population of interest. In addition, although the molecular basis of weak CcEe expression was identified in several samples, a high number of discrepancies between serological test results and genotype results has been observed. For example, while weak E agglutination was found in three samples, genotyping did not reveal any *E* alleles in the same samples.

Overall our findings illustrate the diversity of *RHD* and *RHCE* alleles in the Moroccan population for the first time. Although the performance of routine techniques has evolved, detection of variant phenotype needs complementary serological techniques, such as an indirect antiglobulin test and fixation elution. While these serological tests are still imperfect, genotyping when discrepancy/ambiguity occurs is recommended to guarantee patients' safety. Global knowledge of variants in a specific population is critical for selecting the most appropriate reagents and phenotyping techniques and for developing a dedicated genotyping strategy. This study, as well as the data generated in its course, is definitely a milestone that will help physicians to ensure better prevention of transfusion and foetal-maternal alloimmunisation in the Moroccan population.

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Authorship contributions

HEH, MEW and NH: substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content and final approval of the version to be published.

YF, CF, ZO, FZ, MB, KB, NN and RA: contributions to technical assistance, acquisition of data, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content and final approval of the version to be published.

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