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Transfusion strategy for weak D type 4.0 based on *RHD* alleles and *RH* haplotypes in Tunisia

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Abstract

Background—With more than 460 *RHD* alleles, this gene is the most complex and polymorphic among all blood group systems. The Tunisian population has the largest known prevalence of *weak D type 4.0* alleles, occurring in 1 of 105 *RH* haplotypes. We aimed to establish a rationale for the transfusion strategy of weak D type 4.0 in Tunisia.

Study design and methods—Donors were randomly screened for the serological weak D phenotype. The *RHD* coding sequence and parts of the introns were sequenced. To establish the *RH* haplotype, the *RHCE* gene was tested for characteristic single nucleotide positions.

Results—We determined all *RHD* alleles and the *RH* haplotypes coding for the serologic weak D phenotype among 13,431 Tunisian donations. A serologic weak D phenotype was found in 67 individuals (0.50%). Among them, 60 carried a *weak D type 4* allele: 53 *weak D type 4.0*, 6 *weak D type 4.2.2* (DAR), and 1 *weak D type 4.1.* Another 4 donors had 1 variant allele each: *DVII*, *weak D type 1*, *weak D type 3*, and *weak D type 100*, while 3 donors showed a normal *RHD* sequence. The *weak D type 4.0* was most often linked to *RHCE*ceVS.04.01*, *weak D type 4.2.2* to *RHCE*ceAR*, and *weak D type 4.1* to *RHCE*ceVS.02*, while the other *RHD* alleles were linked to one of the common *RHCE* alleles.

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Authorship contribution: SJY collected the samples and coordinated the blood donor study; MO and HR performed the serologic testing; and MO, HR and SJY analysed the serologic data. MO and KS performed the molecular testing; and MO, KS and WAF analyzed the molecular data. SJY and WAF contributed tools, methods and essential reagents. MO wrote drafts and WAF the manuscript.

Web Resource

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Conclusions—Among the weak D phenotypes in Tunisia, no novel *RHD* allele was found and almost 90% were caused by alleles of the weak D type 4 cluster, of which 88% represented the *weak D type 4.0* allele. Based on established *RH* haplotypes for variant *RHD* and *RHCE* alleles and the lack of adverse clinical reports, we recommend D positive transfusions for patients with weak D type 4.0 in Tunisia.

Introduction

Rh, encoded by the *RHD* and *RHCE* genes, is the most complex and polymorphic blood group system in humans. Apart from ABO,[1] the D antigen is the most immunogenic and clinically significant blood group antigen. Many alleles cause qualitative and quantitative changes in the expression of the D antigen on red blood cells. As a consequence, this diversity of D variants, such as weak D and partial D, causes variable serologic reactivity with monoclonal anti-Ds. The serologic weak D phenotype occurs in 0.2% to 1% Caucasians,[1] while DAR (weak D type 4.2) alone was found in 1.5% of the individuals in an African Black population.[2] The human RhesusBase[3] lists more than 150 molecular weak D types, with weak D types 1 to 3 representing more than 90% of all weak D types in Caucasians.[1] In 2015, a Work Group determined that pregnant women with a weak D type 1, 2 or 3 are not at risk of forming alloanti-D and can safely be exposed to regular D positive red cells.[4] However, the Work Group refrained from determining this risk in individuals with weak D types 4.0 or 4.1 until more data becomes available,[4] because there is a known risk for anti-D alloimmunization in individuals with weak D type 4.2 (DAR). To obtain such data, clinical observations could be gathered in populations with a greater prevalence of such alleles than found in Caucasians.[5]

D antigen variants have been studied at the DNA level in any Arab population since 2009 only, notably in Gaza,[6] Tunisia,[7–19] Egypt,[20–22] and Libya.[23] The molecular causes of the serologic D negative,[7,9,15,16] weak D[7,10–13,15–18] and partial D phenotypes[7,10,17–19] have been evaluated in different sets of Tunisian samples ranging from 100 to 2,000 samples each. Similar to Caucasian populations,[24,25] the prevalence of individuals carrying the *RHD* gene approximated 2.5% among serologically D negative Tunisian individuals[7,15] and 25% among D negative with C+ or E+ antigens.[16] Likewise, the *RHD* gene deletion (*RHD*01N.01*) was most common (97.5%), while *RHD*Ψ* (0.7%) and various *RHD-CE-D* hybrid alleles (1.1%) were rare, but some weak D types (0.7%), including 2 weak D type 4.0 (0.2%), were also found. However, the most striking difference to any previously studied population was the much greater prevalence of *weak D type 4.0* among 1,000 random donors tested using molecular methods,[12] of whom 19 donors carried the *weak D type 4.0* allele, all confirmed by *RHD* sequencing. Hence, the frequency of *weak D type 4.0* was 1 in 105 *RH* haplotypes in Tunisia as compared to 1 in 6,060[26] or less in Europe.[27]

A systematic study was missing for samples with the serologic weak D phenotype routinely found in blood donor and patient testing in Tunisia. We tested a cohort of 13,431 Tunisian blood donations, identified all samples with a serologic weak D phenotype, and sequenced their *RHD* genes. Characteristic single nucleotide polymorphisms (SNP) were also determined to ascertain the *RHCE* allele linked to the known *RHD* allele, thus constituting *RH* haplotypes.

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Materials and Methods

Study subjects

EDTA-anticoagulated whole blood samples from 13,431 random blood donations were collected at the Regional Blood Transfusion Center of Sousse (CRTS) which serves the 4 Gouvernorates Sousse, Monastir, Mahdia and Kairouan of eastern Tunisia between December 2015 and August 2016. Some donors may have donated repeatedly, which is common in previous similar large studies and known to not affect the statistics and conclusions. The study was approved by the Institutional Review Board of the University Hospital Farhat Hached, Sousse, Tunisia (IRB00008931).

Immunohematology

For the D antigen (RH1), we used a monoclonal anti-D reagent (clones P3×61 [IgM], P3×21223B10 [IgM], P3×290 [IgG] and P3×35 [IgG], lot no. AC-125; Biomaghreb, Tunis, Tunisia) and another monoclonal IgG/IgM mixture (lot no. DD1401-y13; Fortress Diagnostics, Antrim, UK). An indirect antiglobulin test was performed in case of negative reactions. For the CEce antigens, we used 1 monoclonal reagent each (all IgM; Bio-Rad, Marnes-la Coquette, France): anti-C (RH2, clone MS24), anti-E (RH3, MS260), anti-c (RH4, MS33) and anti-e (RH5, MS16, MS21, and MS63).

Rh phenotyping was performed in all samples by hemagglutination in 3 techniques (opaline plate, tube, and microtiter plate)[28] according to the regulation for blood donor testing in Tunisia (Circular no. 49). A serologic weak D phenotype[4] was defined by a 2+ or less agglutination strength in any of the 3 methods routinely used (Table S1). A panel of 6 monoclonal anti-D clones LHM76/55 (IgG), LHM77/64 (IgG), LHM70/45 (IgG), LHM59/19 (IgG), LHM169/80 (IgG), and LDM1 (IgM) was used in antiglobulin technique (rabbit polyspecific anti-IgG and monoclonal anti-C3d, clone C139-9) with gel cards (ID-Partial RhD-Typing Set, Bio-Rad).

Red cell genotyping

The *RHD* gene was sequenced in all samples with serologic weak D phenotype and the *RHCE* gene in select samples as described previously.[27,29] The nucleotide sequences of all 10 exons as well as the adjacent intronic regions including the 5′ and 3′ untranslated regions (UTR) were determined. Zygosity testing for the *RHD* gene was done by quantitative fluorescence polymerase chain reaction (QF-PCR) using *RHD* intron 4[24] and *RHCE* exon 7 (two-copy internal control) as described previously.[30] For screening of the *RHCE* gene, characteristic SNPs were determined (RHCE BeadChip Kit, BioArray Solutions),[31] which cannot detect *RHCE*ceAG* recently described in African Americans.[32] Nucleotide sequences were aligned and compared to reference sequences as described previously.[33]

When sequencing of *RHD* exons 5 or 6 or both failed in 5 samples – there was no amplification because of presumably low DNA quality – we were, however, able to confirm the positions 602 (T201R) and 667 (F223V) by PCR-SSP[12,18] and assigned weak D type 4.0: No possible alternative *RHD* allele was known for 2 samples; while *weak D type 4.3*,

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weak D type 4.0.1, and *RHD(T201R,F223V,G307R)*[3] could not be ruled out in 3 samples, such alleles that are rare in Caucasian have never been observed in Tunisia.

Statistics

The 95% confidence interval (CI) for allele frequencies was calculated based on the Poisson distribution using a web resource. The *DVII* allele frequency in Southwestern Germany was calculated from the observed phenotype frequency.[34] *RH* haplotype frequencies are known for Tunisia[35] and Germany.[36] A 2-sided Chi-Square test was performed to compare the *DVII* allele frequency distributions between 2 populations.[19,34]

Nomenclature

The *RHCE*ce(48C, 105T, 733G, 744C, 1025T)*, as observed in Tunisia[12] and France,[37] differs by 2 silent SNPs from the ceTI type 2.[38,39] Per ISBT Working Group on Red Cell Immunogenetics and Blood Group Terminology, alternative names for this *RHCE* allele are *ceTI type 2.01, RHCE*01.20.04.01, RHCE*ce.20.04.01, and RHCE*ceVS.04.01*.

Results

Using blood center routine methods, we screened 13,431 blood donations in Tunisia for the D antigen, 11,974 of whom were found D positive (89.15%) and 1,390 D negative (10.35%). The serologic weak D phenotype was observed in 67 distinct donors (0.50%). They were sorted based on the anti-D agglutination strength in the 3 routine techniques, and 47 of them were also tested with a panel of 6 monoclonal anti-D reagents (Table S1). Despite multiple serologic routine methods were applied, a discrimination of D variant was impossible by serology alone.

RHD alleles

We determined the full length *RHD* coding sequence in all 67 samples (Table 1). Among them, 60 carried an allele of the weak D type 4 cluster (89.6%), of which 53 samples (88.3%) showed the *weak D type 4.0* allele. We deposited 3 representative alleles (GenBank accession no. KY075647 to KY075649) including 106 nucleotides of the 5′ UTR and at least 126 nucleotides of the 3′ UTR for a total of at least 5,019 nucleotides of *RHD* gene (Table 1). Only 1 sample each was found for the weak D types 1, 3 and 100 and the DVII, while 3 samples showed the consensus *RHD* sequence, all compatible with published GenBank data (Table 1).

Comparing allele frequencies with previous Tunisian cohorts

The *weak D type 4.0* was the most prevalent molecular weak D type in Tunisians, confirming our previously published data.[12] Comparing our current data (Table 2), we concluded that only approximately 69% of all Tunisian donors expressing the weak D type 4.0 phenotype (53 out of a calculated 77) were actually typed as having a serologic weak D phenotype. Also, many weak D type 4.0, even if recognized among the serologic weak D phenotypes, were considered D positive for transfusion purposes according to the current routine serology standards in Tunisia (Table S1). Without molecular data, at least 1 weak D type 4.2 carrier would have been assigned as D positive for transfusions (Table S1).

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RHCE alleles

All 67 samples were tested for characteristic SNPs by a DNA bead platform. The *RHCE* exons 1, 5 and 7, harboring diagnostic SNPs, were sequenced in all 53 weak D type 4.0 samples and included in the GenBank submissions (KY075647 to KY075649), indicating the *RH* haplotypes. We tabulated the concordance between distinct *RHD* alleles and distinct *RHCE* alleles along with the frequency of *RH* haplotypes formed by such *RHD-RHCE* linkage disequilibrium in the population (Table 3). All Rh phenotypes (CcDEe) observed were compatible with the prediction derived from red cell genotyping of the involved *RHCE* alleles.

DVII alleles

The greatest prevalance of *DVII* alleles had been reported in the German population. Comparing published data (Table S2),[19,34,36] we found that the *DVII* allele may be more common in the Tunisian (0.4%) than the German population (0.13%). This difference was statistically significant ($p<0.001$, $\chi^2=19.94$, 2-sided).

Discussion

The prevalences of the common weak D types 1, 2, 3, 4, 5 and 11 alleles had been tested moleculary among 2,000 random blood donors in Tunisia including D positive and D negative individuals.[18] Here we identified all samples with a serologic weak D phenotype in a cohort of 13,431 Tunisian blood donations, sequenced their *RHD* genes and established the *RH* haplotypes. The study was designed to obtain data on weak D type 4.0 in a population known to harbor the greatest prevalence of such allele worldwide.[12,18] Recently, a Work Group[4] identified the need for more data for weak D types 4.0 or 4.1,[4] the topic of this research.

Only 2 samples found by our screen for the serologic weak D phenotype represented weak D types 1 and 3 (Table 1), which are much more common in Caucasians. Because we performed molecular genotyping only in cases with serologic weak D phenotype, the true prevalence of D variants in the Tunisian population certainly exceeds 0.50%. An estimated 24 out of 77 Tunisian individuals (31%, Table 2) expressing the weak D type 4.0 phenotype have routinely been typed as D positive, and would eventually be transfused with D positive blood and not receive RhIG in case of pregnancies. No adverse clinical effect has been documented in Tunisia, except 1 observation of an auto-anti-D.[13]

Alloanti-D immunizations have not been observed in weak D types 1, 2, and 3; therefore, carriers of these alleles may safely be transfused with D positive blood.[4,40,41] There is a consensus that pregnant women and recipients of blood transfusions expressing the weak D type 4.2 (DAR phenotype) should be managed as D negative and require anti-D prophylaxis.[2,40] A recent Work Group refrained from a recommendation of how to manage weak D type 4.0 in the US,[4] although a D positive strategy was recommended in Europe[41] and has been recently adopted for Tunisia.[18] The weak D type 4.0 has been associated with low-titer anti-D, often difficult to distinguish between auto- and allo-anti-D. In a recent summary, only 1

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of 16 observations was confirmed as allo-anti-D[4] in France,[42] and no additional examples have been published since.

Based on our previous studies[12] and the current data (Table 2), along with the lack of any observed substantial adverse clinical effect,[13] we conclude that patients and pregnant women in Tunisia expressing weak D type 4.0 phenotype should be treated as D positive and should not be exposed to RhIG, from which these women or their babies cannot be expected to benefit clinically. We propose this strategy as a pragmatic clinical decision in the light of current evidence. If eventually a rare allo-anti-D immunization should occur, a revised strategy may be considered in Tunisia, depending on the frequency, the clinical relevance and the cost to detect and manage weak D type 4.0 with D negative transfusions. Molecular matching studies may determine in the future a clinical significance of some seemingly innocuous protein mismatches even if no allo- or auto-antibody formation would ever occur. Cellular mechanisms might also be involved, and dry matching would forestall such potential, currently hypothetical, clinical issues. There remains a need to monitor, wherever possible, whether patients expressing the weak D type 4.0 phenotype would incur allo-anti-D or other detrimental clinical effects, because our current strategy is based on today's absence of evidence.

The molecular analysis of the *RHCE* gene showed that 59 out of 67 samples with serologic weak D phenotype (88.06%) had a variant *RHCE* allele and the most common associations were: *weak D type 4.0* linked to *RHCE*ceVS.04.01*; and *weak D type 4.2.2* with *ceAR* (Table 3). A previous study conducted in Tunisian[12] and French populations,[37] showed that *weak D type 4.0* is predominantly cis-associated with *RHCE*ceVS.04.01* with rates of 100% and 87%, respectively. Although we frequently found *weak D type 4.0* associated with *RHCE*ceVS.04.01*, the combination of *weak D type 4.0* with *RHCE*ceVS.02* was more prevalent in the Brazilian study (63.4%).[43] Hence, the associated *RHCE* alleles differed to some extent depending on the population studied.

There is a possibility that the *RHCE*ceVS.04.01* allele, typically associated in Tunisian individuals (Table 3), may protect from allo-anti-D immunization, while other *RHCE* alleles, such as the *RHCE*ce* more often associated in individuals of other ethnic groups, may not. This conjecture, supported by not much evidence, would need corroboration by experimental and clinical data before it could be used to guide clinical recommendations.

The *weak D type 100* observed in our study was associated with an *RHCE*ce* allele (Table 3) while it had previously been reported with an *RHCE*Ce* allele.[44] The serologic weak D phenotype in 3 samples (Table 1) without any *RHD* coding exon mutation may be explained by the suppressive effect of the *RHCE*Ce* allele in trans. We observed only 1 donor with a *DVII* allele among the serologic weak D phenotypes, while the *DVII* allele was shown to be more common (Table S2) in the Tunisian (0.4%)[19] than even the German population (0.13%).[34] Similar to weak D type 4.0, most DVII were likely typed as normal D, not having a serologic weak D phenotype. We regularly and unknowingly transfuse most patients carrying the DVII, a partial D, with D positive blood.[45] No precautions to detect DVII have ever been mandated, although D negative transfusions would be recommended in any patient, especially women of childbearing age, known to express DVII, as carriers of this

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partial D can make anti-D. The study exemplified that D variants cannot reliably be discriminated with serologic methods (Table S1)[46–48] using samples from the Arab population, which has been characterized by serology and molecular techniques before (see Supplement).[49–53]

No novel *RHD* allele was found in our study among 67 donors with the serologic weak D phenotype. We conclude that the molecular description of *RHD* alleles may have become rather complete, even for complex and variable alleles, such as represented by the *RHD* alleles constituting the weak D type 4 cluster.[54] While *weak D type 4.0* is the most prevalent weak D type in Tunisia (Table 1),[12] data from Egypt documented *weak D type 4.2* as most prevalent.[20,21] Hence, exploring the distribution of *RHD* alleles remains incomplete for many populations and may yield interesting clinical clues for current questions,[4] as demonstrated by weak D type 4.0 in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

RHD alleles found among donors with a serologic weak D phenotype

	Donors observed			*RHD* allele frequency in the population corrected for *RH* haplotype frequency *		Comment	
***RHD* allele**	**n**	**Rh phenotype**	***RH* haplotype**	**Estimate**	**95% CI**	**ISBT terminology**	**GenBank**
Weak D type 4 cluster	60						
weak D type 4.0	53	ccDee †	*cDe*	1:193 ‡	n.a. ‡	*RHD*09.03.01*	KY075648
weak D type 4.2.2	6	ccDee †	*cDe*	1:1,710 ‡	n.a. ‡	*RHD*09.01.02*	KY075647
weak D type 4.1	1	ccDee	*cDe*	1:10,260 ‡	n.a. ‡	*RHD*09.04*	KY075649
Other weak D types	3						
weak D type 1	1	CcDee	*CDe*	1:12,838	1:3,718 – 1:43,417	*RHD*01W.1*	AJ428455
weak D type 3	1	CcDee	*CDe*	1:12,838	1:3,718 – 1:43,417	*RHD*01W.3*	KF680198
weak D type 100	1	ccDee	*cDe*	1:10,260	1:3,718 – 1:43,417	*RHD*01W.100*	LC053443
Other *RHD* alleles	4						
DVII	1	CcDee	*CDe*	1:12,838 ‡	n.a. ‡	*RHD*07.01*	KC515380
RHD consensus	3	CcDee †	n/a	n.a.	n.a.	*RHD*01*	NG_007494.1
Total	67						

*Corrected for the *RH* haplotype frequency and *RHD* allele variants masked by normal D positive *RHD* alleles in trans.

†2 of the 53 donors were CcDee, 1 of the 6 donors was compound heterozygous for *weak D type 4.2.2/RHD*Ψ*, and 2 of the 3 donors CCDee.

‡The DVII frequency is known to be much greater (see Table S2), as most carriers of DVII occur among D positive donors and do not present a serologic weak D phenotype. To a smaller extent, this caveat also applies to alleles of the weak D type 4 cluster. For instance, the frequency of *weak D type 4.0* is actually 1:105, as determined by PCR in random donors.[12]

n.a. – applicable

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Table 2

Prevalence of Tunisian individuals expressing the weak D type 4.0 phenotype *

		Frequency as published		
Parameter	**Number of donors**	**Rate**	**Fraction**	**References and calculations**
Donors (all)	13,431	n/a	n/a	This study
RhD antigen negative *RH* haplotypes	n/a	0.3021	1 : 3.31	*cde* = 0.284, *Cde* = 0.018, *cdE* = 0.0001 [36]
Weak D type 4.0 positive *RH* haplotypes	n/a	0.0095	1 : 105.26	2,000/19 = 105.26 †[12]
Donors with weak D type 4.0 phenotype *				
Expected (calculated)	77	n/a	n/a	13,431 × (0.3021 × 0.0095) × 2 = 77.25 ‡
Observed (*RHD* sequence confirmed)	53	n/a	n/a	This study

*These individuals must be hemizygous for an *RH* haplotype with a functional *RHD* allele on 1 chromosome (i.e., no functional *RHD* allele on the other chromosome).

†Among 1,000 random donors, including RhD negative donors and representing 2,000 *RH* haplotypes, a total of 19 donors were found carrying a *weak D type 4.0* allele.[12]

‡The frequency of an RhD antigen negative *RH* haplotype paired with the *weak D type 4.0* positive *RH* haplotype must be multiplied by 2, because this haplotype combination can occur in 2 ways for the pair of chromosomes in a given donor.

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Table 3

RHCE alleles linked to *RHD* alleles (*in cis* on 1 chromosome)

		***RHCE* allele linkage**		
***RHD* alleles**	**Donors observed (n)**	***RHCE* alleles observed**	**n**	***RHCE* allele probable or proven *in cis* to *RHD***
Weak D type 4 cluster	60			
weak D type 4.0	53	*RHCE*ce + RHCE*ceVS.04.01*	43	*RHCE*ceVS.04.01*
		*RHCE*ce.01 + RHCE*ceVS.04.01*	2	*RHCE*ceVS.04.01*
		*RHCE*Ce + RHCE*ceVS.04.01*	2	*RHCE*ceVS.04.01*
		*RHCE*ceVS.02 + RHCE*ceVS.04.01*	2	*RHCE*ceVS.04.01*
		*RHCE*ceVS.04.01 + RHCE*ceVS.04.01*	2	*RHCE*ceVS.04.01*
		*RHCE*ce + RHCE*ceVS.02* or *RHCE*ce.01 + RHCE*ceVS.01*	1	*RHCE*ce*
		*RHCE*ce + RHCE*ce*	1	*RHCE*ce*
weak D type 4.1	1	*RHCE*ce + RHCE*ceVS.02*	1	*RHCE*ceVS.02*
weak D type 4.2.2	5	*RHCE*ce + RHCE*ceAR*	5	*RHCE*ceAR*
*weak D type 4.2.2/RHD*Ψ*	1	*RHCE*ce.01 + RHCE*ceAR*	1	*RHCE*ceAR*
Other weak D types	3			
weak D type 1	1	*RHCE*Ce + RHCE*ce*	1	*RHCE*Ce*
weak D type 3	1	*RHCE*Ce + RHCE*ce*	1	*RHCE*Ce*
weak D type 100	1	*RHCE*ce + RHCE*ce*	1	*RHCE*ce*
Other *RHD* alleles	4			
RHD consensus	3	*RHCE*Ce + RHCE*Ce*	2	*RHCE*Ce*
		*RHCE*Ce + RHCE*ce*	1	*RHCE*Ce*
DVII	1	*RHCE*Ce + RHCE*ce*	1	*RHCE*Ce*
Total	67		67	

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