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# *RHD* genotyping and its implication in transfusion practice

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ABSTRACT

*Background:* The limitations of serology can be overcome by molecular typing. In order to evaluate the contribution of RH systematic genotyping and its implication in transfusion practice, a genotyping of D– blood donors was initiated.
*Methods:* Blood samples were collected from 400 unrelated D– individuals. All samples were tested by *RHD* exon 10 PCR. In order to clarify the molecular mechanisms of *RHD* gene carrier, we applied molecular tools using different techniques: PCR-multiplex, and PCR-SSPs.
*Results:* Among 400 D– subjects tested, 390 had *RHD* gene deletion; and 10 had *RHD* exon 10 of which seven were associated with the presence of the C or E antigens. Among D– carriers, we observed in five cases the presence of *RHD-CE-Ds* hybrid, in four cases the presence of pseudogene *RHD* $\psi$ and in one case the presence of weak D type 4.
*Conclusion:* Since the majority of aberrant alleles were associated with C or E antigens and the preliminary infrastructure for molecular diagnostic were absent in all Tunisia territory, we recommend to reinforce transfusion practice to consider D– donors but C+/E+ antigens as D+ donors and the application of *RHD* molecular typing only to solve serologic problems.



## 1. Introduction

The D blood group antigen is the most important protein of the Rh system due to its involvement in hemolytic disease of the fetus and newborns (HDFN) and in hemolytic transfusion reactions (HTR). Anti-D remains the most common cause of HDN despite the use of anti-D prophylaxis in D– women. Approximately 80% of D– healthy volunteers transfused with 1 or more D+ blood units produce anti-D [1]. More recent data showed that only 20%–30% of patients transfused with 1 or more D+ units produce anti-D [2–4], Consequently, To prevent anti-D alloimmunization, exposure of D– individuals to D+ red blood cells (RBCs) should be avoided by appropriate transfusion strategies, and routine administration of anti-D immunoglobulin (Ig) to D– women during the third quarter of pregnancy and after delivery of a D+ infant. The frequency of D– phenotype was estimated at 9% in Tunisia [5]. The D negative phenotype is characterized by a high molecular diversity which explains the discrepancies found between serologic and molecular methods. Approximately 0.4% of the Central European D+ population carries *RHD* alleles associated with reduced D antigen expression [6]. There are evidences that some RBC units with weak D or Del phenotype may escape detection by standard serologic methods including the indirect antiglobulin test (IAT) and may cause anti-D immunization when transfused to D– recipients. Recipients who carry the weak D types 1, 2, 3 and 4.1 can be transfused with D+ RBC units without anti-D alloimmunization. This alloimmunization is documented only for weak D types 4.2 (or DAR), 11 and 15 [7–9]. Geraty (USA) have estimated that in southern California alone each year the red cells from at least 120 weak D or Del donors, typed D– serologically, are transfused to D– recipients [10]. In a study realized by Flegel et al., among 46.133 serologically D– donors, *RHD* genotyping revealed 96 D– samples who carried

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the *RHD* gene. Almost half of these harbored *RHD* alleles expressing Del phenotypes [11]. Also, in our laboratory, we have identified two weak D type 11, 1 weak D type 4.0, 1 weak D type 29, 1 partial DBT individual mistyped as D– by serological tests [12]. So, to overcome the limits of serologic methods, unable to distinguish the phenotypic subtleties of the RH system, the application of *RHD* molecular analysis becomes a precious tool in immuno hematology laboratories in order to resolve serologic difficulties. Therefore, in the present study, we have screened routinely 400 serologically D– donors for the presence of the *RHD* gene in order to evaluate the implication of *RHD* genotyping in transfusion practice in our blood service and thus its contribution in transfusion safety.

## 2. Material and methods

### 2.1. Blood donors

EDTA blood samples were collected at the Regional Blood Transfusion centre of Sousse from 400 Tunisian blood donors characterized as D negative in routine typing for subsequent molecular characterization.

### 2.2. Serologic typing

All samples were tested by hemagglutination in opaline plate with Diagast (Loos, France) and Biomaghreb (Tunis, Tunisia) (each containing P3 × 61 + P3 × 21223B10 + P3 × 290 + P3 × 35 clones). Bio-Rad (Marnes-la Coquette, France) reagents were used to test the following specificities: anti-C (RH2, clone MS24), anti-E (RH3, clone MS260), anti-c (RH4, clone MS33) and anti-e (RH5, clones MS16, MS21, MS63) according to the manufacturer's instructions. The samples were further examined routinely by indirect antiglobulin test (IAT) to detect some weak D variants. The IAT was realized by tube and gel matrix testing (Diamed, France).

### 2.3. Molecular analysis

#### 2.3.1. Genomic DNA extraction

DNA was isolated from peripheral blood for all samples by the salting-out method described by Miller et al. [13] and quantified by optical density measurement with Nanodrop 1000 (Nanodrop Technologies, Wilmington, DE, USA). DNA was further analyzed according to the adopted molecular work-up detailed in the flowchart (Fig. 1).

#### 2.3.2. Simplex PCR for exon 10 detection

All DNA samples were screened individually for the presence of *RHD* exon 10 to detect the deletion or presence of *RHD* gene. This amplification was performed using a pair of primers re 91 and rr 4 [14]. The β-actine gene (207 pb) was included as an internal control using a pair of primer (BACTs 5′CCTTCCTGGGCATGGAGTCCTG3′ and BACTas 5′GGAGCAATGATCTTGATCTTC3′) [15]. The PCR procedure was performed in a final volume of 25 μL with 25 ng of DNA, 0.2 mM dNTPs, 0.2 μmol/L for RH primers and internal control primers, 2.5 mM of MgCl2 and 1U of Taq polymerase. The reaction were realized in the Gene amp® thermocycler (PCR system 9700) using the following conditions: 5 min initial denaturation at 95 °C; 32 cycles of 1 min at 95 °C, 1 min at 60 °C, and 45 s at 72 °C; and 5 min final extension at 72 °C. The PCR products were visualized on a 2% agarose gel after electrophoresis in Tris acetate EDTA buffer.

#### 2.3.3. Multiplex polymerase chain reaction analysis of RHD gene

Samples that were positive for *RHD* exon 10 were further investigated for the presence of *RHD* exons 3, 4, 5, 6, 7, and 9 under previously reported conditions [16]. To avoid false-negative results, an internal control amplifying a 429 bp segment of the human growth hormone gene was included [17]. Multiplex PCR amplicons were controlled on 3% agarose gel.

### 2.4. Molecular characterization of samples that were negative for some exons by multiplex PCR.

#### 2.4.1. PCR-SSPs to detect the d(C)ce$^s$ haplotype

The samples showing the presence of *RHD* exon 9 by PCR-multiplex were further analyzed by PCR-SSPs to screen the *RHD-CE* exon 3 and to detect the following mutations 733C>G and 1006 G>T in exons 5 and 7 of *RHCE* gene as previously described [18].

#### 2.4.2. Multiplex PCR targeting pseudogene (RHD ψ)

Samples negative for *RHD* exon 5 and positive for the other exons by multiplex PCR were typed for the presence of the 37-pb insertion at the intron 3/exon 4 and the *RHD* exon 6 T807G nonsense mutation boundary present in *RHD ψ* [19]. Amplification was carried out in a final volume of 25 μL containing 2.5 g genomic DNA, 3.5 mM of dNTPs, 0.3 U of *Taq polymerase*, 2.5 mM of MgCl2 and 1 μmol/L for each RH primer and for internal control primers. Thermocycling and electrophoresis conditions were identical to the simplex exon 10 PCR procedure.

#### 2.4.3. PCR-SSPs to research the weak D type 4

Samples showing negativity for *RHD* exon 4 and exon 5 by multiplex PCR suggest the eventual presence of two D variants: weak D type 4 or DVI type I. So, to determine the molecular background of these variants, we have used the weak D type 4 genotyping by two PCR-SSPs specific for *RHD* exon 4 C602G mutation and exon 5 T667G mutation as previously described [20].

### 2.5. Calculation of allele frequencies

In our survey, allele frequencies of each molecular background found among the D Tunisian blood donors were calculated by applying the Berstein formula [21].

## 3. Results

Serologic testing of the D– donor pool revealed the following phenotypes: Cc+ E– e+ ($n = 362$), C+ c+ E– e+ ($n = 26$), C– c+ E+ e+ ($n = 10$), C+ c+ e+ E+ e+ ($n = 2$). The screening of weak D variants by IAT was negative for all samples.