RHD alleles in the Tunisian population

Mouna Ouchari, Saloua Jemni-Yaacoub, Taher Chakroun, Saida Abdelkefi, Batoul Houissa, Slama Hmida

Abstract:
Background: A comprehensive survey of RHD alleles in Tunisia population was lacking. The aim of this study was to use a multiplex RHD typing assay for simultaneous detection of partial D especially with RHD/RHCE deoxyribonucleic acid (DNA) sequence exchange mechanism and some weak D alleles. Materials and Methods: Six RHD specific primer sets were designed to amplify RHD exons 3, 4, 5, 6, 7 and 9. DNA from 2000 blood donors (1777 D+ and 223 D-) from several regions was selected for RHD genotyping using a PCR multiplex assay. Further molecular investigations were done to characterize the RHD variants that were identified by the PCR multiplex assay. Results: In the 1777 D+ samples, only 10 individuals showed the absence of amplification of exons 4 and 5 that were subsequently identified by PCR-SSP as weak D type 4 variants. No hybrid allele was detected. In the 223 D-, RHD amplification of some exons was observed only in 5 samples: 4 individuals expressed only RHD exon 9, and one subject lacking exons 4 and 5. These samples were then screened by PCR-SSPs on d(C) ce and weak D type 4, respectively. Conclusion: The weak D type 4 appears to be the most common D variant allele. We have not found any partial D variant. Findings also indicated that RHD gene deletion is the most prevalent cause of the D- phenotype in the Tunisian population.

Key words: Partial D, polymerase chain reaction multiplex, polymerase chain reaction with sequence-specific priming, RHD genotyping, Tunisia, weak D

Introduction

The Rhesus blood group which named 004 by International Society of Blood Transfusion (ISBT 004) is the most complex and immunogenic of the blood group systems. It comprises 50 antigens numbered RH1 to RH57 by the International Society of Blood Transfusion.[1] The most important ones in transfusion and obstetrical settings are D (RH1), C (RH2), E (RH3), c (RH4), and e (RH5). This system is of clinical importance because it is involved in haemolytic transfusion reactions, autoimmune hemolytic anaemia, and haemolytic disease of the newborn (HDN). In fact, 50% of HDN cases are caused by maternal anti-D immunoglobulin G (IgG) antibodies crossing the placenta, that bind to fetal red blood cells (RBCs) followed by their destruction, causing anaemia.[2-4] The antigens of the Rh system are encoded by two highly homologous genes with 97% of similarity, RHD and RHCE.[5] These genes are separated by about 30 kb DNA sequence containing the small membrane protein 1 (SMP1) locus.

It has been reported that among Europeans carrying the RHD locus, 1 percent produces a weak or a partial D.[6] In this regard, several partial D have been described at both phenotypic and molecular levels.[7] Frequencies of D0, D1, D2, D3, D4, and DFR are 1:900, 1:6,800, 1:10,000, 1:30,000, 1:30,000, and 1:60,000, respectively, as established by serological methods in a white population.[8]

The prevalence of RHD alleles differs considerably among ethnic groups. Variant D are known to be frequent in some population, like African persons, whereas they are considered rare in European persons. In Tunisia, RHD alleles have not been fully characterized. The purpose of this study was to perform a systematic study at the molecular level in 2000 blood donors collected at two blood transfusion centers: Centre Regional de Transfusion Sanguine (CRTS) and Centre National de Transfusion Sanguine (CNTS) to determine the frequency of RHD alleles especially with RHD/RHCE hybrid gene present in Tunisian population. It is important to establish the frequency of weak D and partial D because of their impact on transfusion strategies and management of pregnancies. This study could provide information to resolve immunohematology difficulty.

Materials and Methods

Blood samples
Ethylene diaminetetraacetate-anticoagulated blood samples were collected from random blood donors in Tunisia between October 2010 and September 2011. Two blood transfusion centers participated in this study: CRTS of Sousse (1000 samples: center) and CNTS of Tunis capital
(1000 samples: north and south). One sample from CNTS (weak D type 4.0) was used for validation of PCR multiplex.

Serologic testing
Serologic testing was evaluated by hemagglutination with two commercially available monoclonal antibody (MoAb) reagents from Diagast (Loos, France) and Biomaghreb (Loos, France). The reagent was prepared from a blend of both IgG and immunoglobulin M (IgM) anti-D. Diagast and biomaghreb reagents were used to test the following specificities: anti-D (RH1, clones P3x61 + P3x21223B10 + P3x290 + P3x35). Bio Rad reagents (France) were used to test the following specificities: anti-C (RH2, clone MS24), anti-E (RH3, clone MS260), anti-c (RH4, clone MS33), anti-e (RH5, clones MS16, MS21, MS63). Phenotype of the red blood cell (RBCs) was performed according to the manufacturer’s instructions. We have added an indirect antiglobulin test (IAT) if they were negative with anti-D reagents in the direct agglutination tests.

Molecular analysis and RHD genotyping
DNA was isolated by a modified salting-out procedure following the technology described by Miller et al[9] and quantified by optical density measurement with Nanodrop 1000 (Nanodrop Technologies, Wilmington, DE, USA). DNA was further used to identify aberrant RHD alleles.

Multiplex PCR analysis
PCR analysis to reveal partial D and some weak D alleles was performed by amplifying RHD exons 3 through 7, and 9 with RHD sequence specific primers, as described by Maaskant-van Wijk et al.[Table 1][10]. The multiplex PCR conditions used in this study were slightly modified from those described by Maaskant-van Wijk et al.[10] Each 25 µl PCR reaction mixture contained 250 ng of DNA, 0.2 mmol/L of dNTPs, 2 units of Taq DNA polymerase (Dream Taq™ DNA polymerase, Fermentas), 3.5 mmol/L of MgCl₂, and primer sets at different concentrations [Table 1]. A pair of nucleotides was used to generate a 429 pb PCR fragment from the human growth hormone gene [Table 1][11] which was included as an internal control to avoid false negative results. Amplification was performed in a thermocycler (Gene Amp® PCR System 9700). After an initial denaturation at 94°C for 2 min, a total of 32 cycles were carried out using the following sequence: denaturation at 94°C for 3 min, primer annealing at 60°C for 1 minute, polymerization at 72°C for 45 s, and one cycle at 72°C for 5 min to complete extension.

Specific products range from 157-57 bp and were visualized on 3-percent agarose gels and visualized by ethidium bromide staining.

PCR-SSPs for RHD variants
• Based on classification criteria of Maaskant-van Wijk et al[10] the lack of 126 (exon 4) and 157 (exon 5) PCR bands was associated with D<sup>1</sup> type 1 genotype. But, a gene for a D<sup>1</sup> type 1 variant would give the same result of weak D type 4 allele. In order to determine the molecular background of these variants, we used the weak D type 4 genotyping by PCR-SSP. This technique was devised to detect or to confirm the T201V (C602G) and T223V (T667G) substitutions in the weak D type 4 alleles and triggered to work under similar PCR conditions as PCR SSP system previously developed for RHD typing.[12,13] Specific primers were gw44a and primer gw44b for T201V [Table 2][13] generating a 138 bp. Specific primers were Rh223vf and primer ga51 for T223V [Table 3][13] generating a 164 pb. To avoid false negative results, an internal control amplifying the human growth hormone gene (429 pb) [Table 1][11] was included. Amplifications were carried out with Taq (Invitrogen) in a final volume of 10 µL. The reactions worked under similar PCR conditions on a DNA thermocycler (Gene Amp® PCR System 9700). Cycling conditions consisted of an initial denaturation of 2 min at 94°C, followed by ten cycles of 10 s denaturation at 94°C and 1 minute annealing/extension at 65°C, and finally 22 cycles of 30 s denaturation at 94°C, 1 minute annealing at 61°C and 30 s extension at 72°C. After amplification 5 µL of the final PCR products were analyzed on 2% agarose gels.

• Samples positive for only RHD exon 9 have been further analysed by PCR-SSP to screen for the D-CE hybrid exons 3 and PCR-ASP to detect the following mutations C733G and G1006T in exon 5 and 7 of RHCE. All primers were the same as those used by Daniels et al. [Table 2][15] For detection of the presence of a D-CE hybrid exon3, primer RHD-EX3F, which anneals to an RHD specific sequence at the 5′ end of exon 3 (T380, A383), was paired with RHCE-EX3R, a primer specific for the 3′ end of RHCE exon 3 (C455), to amplify a 110 pb product only when RHCE-derived exon 3 is paired with the 5′ end of an RHD-derived exon 3. The control primer (RH-EX7F and RHCE EX7R) amplified a 130 pb product from exon 7 of RHCE. 35 cycles of PCR was performed at 94°C for 30 s, 65°C for 30 s, and 72°C for 45 s. The RHCE-INT4F/RH-EX5CR, RH-EX5GR primer sets were applied to detect C733G

| Primer | Primer direction | Sequence | Position | RHD exon | Size (pb) | Final concentration nmol/L |
|--------|------------------|----------|----------|----------|----------|-----------------------------|
| R364   | Forward          | 5'tCGGTGCTGATCTCAGTGGAG3' | 364-383 | 3         | 111      | 81.1                         |
| R474M  | Reverse          | 5'ACTGATGACCATCCTCATG3' | 455-474 | 126       | 54.7     | 82.6                         |
| R496   | Reverse          | 5'CACTAGAACATGATGCAC3' | 496-514 | 4         | 126      | 48.7                         |
| R621   | Reverse          | 5'CAACCTGGTGATCTGCGATG3' | 602-621 | 6         | 125      | 125                          |
| R648   | Reverse          | 5'TGTTATGCTGATCTCAG3' | 648-667 | 5         | 157      | 125                          |
| Rex5A2D | Reverse           | 5'caCTTTGCTGATCTTACG3' | 787-801 | 5         | 125      | 125                          |
| R898M  | Reverse          | 5'TGATGCGTGCTGATCTCAG3' | 898-916 | 6         | 57       | 85.08                        |
| Rex6A3D | Reverse           | 5'gtagtgatctacGGGCAATG3' | 932-939 | 7         | 96       | 82.1                         |
| R973   | Reverse          | 5'AGCTCTAGATGGGCTACAA3' | 973-992 | 7         | 96       | 82.1                         |
| R1068  | Reverse          | 5'ATTGGCCGCGCTGAGGCTAT3' | 1048-1068 | 9       | 71       | 78                           |
| Rex9SD2 | Reverse           | 5'aacagGTTGGCTCTCAAATTG3' | 1154-1170 | 9       | 71       | 175                          |
| R1219M | Reverse          | 5'AAACTTTGATGATATTTAATCTT3' | 1193-1219 | 9       | 71       | 175                          |
| HGHF   | Forward          | 5'SCCTTCCACACTTCTCCCTA3' | 666-686 | 429      | 100      | 100                          |
| HGHFR  | Reverse          | 3'TCACGGATTTCTGTTGTGGTC5' | 1098-1077 | 4         | 125      | 100                          |

Table 1: Sequences and positions of primers on RHD[13,14]

RHD: gene RHD, pb: paires de base, HGF: human growth hormone gene, HGHF: human growth hormone gene (Forward Primer), HGHFR: human growth hormone gene (Reverse primer)
polymorphism in exon 5. The forward primer (RHCE-INT4F) anneals to an RHCE-specific region of intron 4. The reverse primer is specific for C733 (RH-EX5GR) in one reaction and G733 (RH-EX5CR) in the other. These reactions provide a 428-bp product when the appropriate RHCE exon 5 sequence is present. The positive control was a 130-bp fragment of the RHCE exon 7. The extension reaction included 30 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 5 s, and extension at 74°C for 30 s. For the G1006T polymorphism in RHCE exon 7, two separate PCR-SSPs were carried out, each employing two pairs of primers. One forward primer was specific for either G1006 (RH-EX7GF) or T1006 (RH-EX7TF), its reverse partner (RHCE-EX7R) was specific for an RHCE specific sequence within exon 7. These primers amplified a 94-bp product when the appropriate RHCE exon 7 sequence was present. In each PCR procedure, the positive control was a 429-bp fragment of the human growth hormone gene [Table 1].[11] 30 cycles of PCR were performed at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

**Table 2: Oligonucleotide primers used for PCR-SSPs**[16-18]

| Primer    | Primer direction | Sequence | RHD/RHCE Exons | Final concentration nmol/L |
|-----------|------------------|----------|----------------|---------------------------|
| RHCE-INT4F | Forward          | 5’ GCAAACAGCGCAAGATGCAA3’ | RHCE Intron 4 | 100                        |
| RH-EX5GR  | Reverse          | 5’TGTGACAACACTGAGCTGA3’ | RHCE Exon 5  | 100                        |
| RHCE-EX7R | Reverse          | 5’ACCAAGTCGACTGCCGTC3’ | RHCE Exon 7  | 100                        |
| RH-EX7F   | Forward          | 5’ACCCACATGCCATTGCCGTTC3’ | RHD Exon 3   | 200                        |
| RH-EX7GF  | Reverse          | 5’TCTGCTCACCTTGCTGATCTTCCC5’ | RHD Exon 4   | 200                        |
| RH-EX7TF  | Forward          | 5’TGGTGGGTGATCGCTAGTGGAA3’ | RHD Exon 5   | 100                        |
| RH-EX3F   | Reverse          | 5’TGTGAGATGAGCATCCTCAGGG3’ | RHEX5GR      | 200                        |
| RHCE-EX3R | Reverse          | 5’TGTCACCACACTGACTGCTAG3’ | RHCE Exon 7  | 100                        |
| gwd4a     | Reverse          | 5’CAGACAAACTGGGTATCGTTGCTC5’ | RH-EX5CR     | 200                        |
| gwd4b     | Reverse          | 5’ACCCACATGCCATTGCCGTTC3’ | RH-EX7GF     | 100                        |

PCR-SSPs: polymerase chain reaction with sequence-specific priming, RHD: gene RHD, RHCE: gene RHCE, RH: Rhesus

**Results**

The RH phenotype of all 2000 blood donors has been determined by the routine serological method. We have identified 1777 samples D+ (88.85%), and 223 samples D- (11.15%). Based on the number of donors observed, we have calculated the Rhesus phenotype frequency [Table 3].

In this study, we have adopted a multiplex PCR method to screen RHD alleles in our population. RHD specific exons 3, 4, 5, 6, 7, and 9 were amplified simultaneously.

From all DNA samples, the 429 bp internal control fragment from the 2000 samples was amplified. The sample used for validation has shown the expected profile. Thus, the bands corresponding to the RHD exons 4 and 5 were absent in weak D type 4.0 sample [Figure 1].

According to the multiplex PCR results, we identified only 15 aberrants RHD alleles in 2000 blood donors [Figure 2]. Among 1777 D+ samples, RHD exons (3, 4, 5, 6, 7, and 9) were generated in 1767 (99.44%). The 10 other samples (0.56%) were negative for RHD exon 4 and 5 [Figure 1] carrying T201V (G602G) [Figure 3-a] and Rh223vf (T667G) substitutions [Figure 3b] which characterize the weak D type 4. All these variants were associated with ccDee phenotype. Among 223 D- samples, 218 individuals (97.76%) were negative for the RHD exons suspecting an RHD deletion which were in accordance with the serological results.

4 samples (1.79%) were only positive for RHD exon 9 that suspect an RHD hybrid allele. Further investigations by SSP-PCR, for these samples have shown a positivity for D-CE exon 3 [Figure 4a] and an heterozygous level for RHCE polymorphisms [Figure 4b and c]. These results exhibited a d(C) ce haplotype. Also, we have identified one weak D type 4 (0.45%) in serologically D- individual which has been missed by indirect antiglobulin test in our study.

**Discussion**

The frequencies of phenotypes D+ and D− are 88.85% and 11.15%, respectively. The most common phenotypes of serological D+ individuals in our group were CcDee, cCDdee, and ccDee. Also, the ccdDee phenotype was the most frequent in the D− samples. These results were in accordance with the previous study in our population.[16] Several methods have been described for RHD genotyping; Benet et al[17] have used sequence differences in the 3’ NCR of RHD and RHCE. Arce et al[18] have developed an RHD assay based on the fact that RHCE intron 4 is larger than RHD intron 4. Sequence differences in exon 4 and 7 have been also used.[19,20] Because the structure of RHD gene is so complex and the genetic diversity evidenced underlying D people, it is necessary to use more than one pair of primers to prevent false negative and false positive results.[21,22]

In this study, we have adopted a multiplex PCR method to analyze the RHD gene profiles of Tunisian population. This technique is based on amplification of the RHD specific exons 3, 4, 5, 6, 7, and 9 in one reaction mixture. RHD exons 1, 2, and 8 and the coding region of exon 10 are equal to the corresponding RHCE exons, and their amplification is therefore not specific for RHD. This method seems to be a good complement of serologic D typing techniques and may reduce the occurrence of anti-D alloimmunization. Except for D[19], all qualitative RHD variants (total or partial replacement of an RHD exon by its RHCE equivalent) known at present, can be recognized by this multiplex PCR technique. D[19] is caused by the replacement of RHD exon 2 with RHCE; RHD exon 2 cannot be specifically amplified. The assay described by Maaskant van Wijk et al[10] also allowed identification of some weak D alleles (weak D types 4.0, 4.0.1, 4.1, 4.2.1, 4.2.2, 4.2.3, 4.3, 14, 29, 40, and 51) according to alternative nucleotide changes.
Table 3: Phenotyping results of 2000 donors

| Phenotypes  | Number | Frequencies (%) |
|-------------|--------|-----------------|
| CcDee       | 728    | 36.4            |
| CCDee       | 422    | 21.1            |
| ccDee       | 230    | 11.5            |
| CcDee       | 182    | 9.1             |
| CcDDee      | 182    | 9.1             |
| CCDee       | 26     | 1.3             |
| CcDDee      | 198    | 9.9             |
| CcdDee      | 19     | 0.95            |
| ccdDee      | 3      | 0.15            |
| ccdDee      | 2      | 0.1             |
| CcDDee      | 1      | 0.05            |
| Total       | 2000   | 100             |

Mutations characteristic of these variants involve the RHD specific nucleotides at the 3’ end of the primers used in this multiplex PCR assay. This is a very reliable technique for the detection of partial D especially with RH/D-RHCE DNA sequence as well as some weak D alleles. The absence of amplification of some exons were associated with several types of variants; exon 3 with Diiic, DiiV type 4 and DKK; exon 4 with DFR, DFR-2, DFR-3, weak D type 14, 40 and 51; exon 5 with DiiV, DiiV type 1, 2, 3, 6 and 7, DCs-1, DFV, DTO, DOL, DOL-2, DOL-3, DAU-5, DBS-1, DBS-2, and weak D type 29; exons 3, 4 and 5 with Diiib, Diiii type 5, Diiii type 6, Diiii type 7 and DiiV type 4; exon 3 and 4 with DFR type 5; exons 3 and 7 with DiiVa and DiiVb-1 variants; exons 7 and 9 with DiiVb variant; and 4 and 5 with DiiV type I, weak D type [4.0, 4.01, 4.1, 4.2, 4.2, 4.22, 4.23, 4.3]. Variants DiiV type II and III can be recognized by the multiplex PCR because of the lack of amplification products from RHD exons 4-6 and 3-6, respectively. DBT type I and type II can be also identified by the absence of amplification of exons 5-7 and 5-9, respectively. In RhHar, a segmental exchange between the RHCE and RHD genes resulting in essentially a RHCE gene with a RHD exon 5 occurs. It is possible that a double crossing-over event rather than gene conversion generates this phenotype. This variant may shown to be negative with all aspects of the multiplex assay except for exon 5.

Rh:-1 subject, RHD alleles like pseudogene RHDΨ and d(C) ce can be recognized by this technique. The RHDΨ is characterized by a 37 bp duplication of the intron 3/exon 4 boundary causing a frame shift and a premature translation termination codon in exon 4, G609A, G654 C and T667G missense mutations in exon 5 and G674T missense and T807G nonsense mutations in exon 6. The d(C) ce have a RHD allele characterized by the presence of RHCE exons starting from nt. 455 (exon 3) to exon 7. PCR multiplex performed on the RHDΨ showed the same profile as partial DiiV (absence of exon 5). However, we obtained only the amplification of exon 9 for d(C) ce.
The multiplex PCR assay technique used in this study is based in simultaneous amplification of six RHD exon fragments. Agarose gel electrophoresis of multiplex PCR products revealed six easily distinguishable bands in D+ and lack all RHD exons in D-. An abnormal PCR profile suggests the presence of a partial D or weak D variant. However, interpretation of the PCR is subject to several pitfalls. Similar profiles can be observed for different D variants, for example, weak D type 4.0 can resemble D41 type I (RHCE like segment of E allele encompassing exons 4-5). The reverse primer of RHD exon 4 and the forward primer of RHD exon 5 are specific for, respectively, C602G and T667G nucleotides substitutions which characterize weak D type 4. The breakpoints of D41 type I are located at the border of intron 5 and exon 6 in a nucleotide range of 215 bp between -100 bp and +115 bp relative to the first nucleotide of exon 6. The conversion point of D41 type I had to be 5 of the intron 3 deletion (288 pb). Because of these pitfalls, abnormal multiplex PCR profiles must be confirmed using another type of tool such as simplex, PCR-SSP or PCR-hybrid allele. That is why in our study we have used a PCR-SSP to characterize the RHD variants that were identified by the PCR multiplex assay.

In our population, 15 aberrants RHD alleles were identified: 10 in D+ and 5 in D− blood donors. Among 1777 D+, we have identified only 10 weak type 4 alleles which were associated with ccDe phenotype. Such association was consistent with published data. It was reported that the allele weak D type 4 allele is associated with ce allele of RHCE gene. The weak D type 4 allele was originally reported to have a C602G and T667G substitutions, respectively, in exon 4 and 5. Other alleles with these changes were subsequently identified and designated weak D types 4.0, 4.0.1, 4.1, 4.2.1, 4.2.2, 4.2.3, 4.3 as they had additional alterations. In our case, it should be noted that the sequencing is necessary to know exactly weak D type 4 alleles.

In the 223 D− individuals, we have identified 4 d(C) ce and one weak D type 4. These findings indicated that in D− individuals the RHD gene deletion was more frequent than the presence of aberrant RHD alleles which the following frequencies: for RHD gene deletion (97, 75%), for d(C) ce (1.8%) and weak D type 4 (0.45%). The presence at heterozygous level of haplotype d(C) ce in our population could lead to the possible presence of this haplotype at homozygous level and then possible existence of RH: 34 phenotype. Our results showed that we are close to Caucasians with a low African contribution due to the d(C) ce haplotype. In our study, we have identified one weak D type 4 in D− individual with a Ccddee phenotype that have been missed by serological tests. This false negative serological result is clinically relevant since transfusion of blood from this D− donor to D− recipient can increase allo-anti-D immunization level. It showed that most, if not all, weak D phenotypes carried altered RhD proteins and anti D alloimmunization was reported in 1 weak D type 15 and 1 weak D type 4.2.2. That is why it is controversial how to transfuse individuals with weak D phenotype. The omission of weak D type 4 in D− individual was also revealed in congo-Brazzaville study showing that 7 of the 110 D− (6.36%) were weak D type 4 alleles (3 weak D type 4.2.2, 1 weak D type 4.0.1, 1 weak D type 4.2.3, 1 weak D type 4.0.1/RHD-CE-Ds, 1 Dar/RHD-CE-Ds). The antigen density of weak D type 4 alleles varied between 1687 and about 4000 RhD antigens per cell. The reduction of the antigen density by a Cde haplotype in trans, was also effective in weak D. So, this false negative result in our case (weak D type 4 associated with Ccddee phenotype) may very well be related to suppressive effect induced by the dCe haplotype in trans. In addition, all weak D type 4 detected in D+ samples was associated with ccDe phenotype that is further proof to the reduction of the antigen density for the weak D type 4 in D-sample by a Cde haplotype in trans.

In our study, no allele with RHD/RHCE hybrid gene was detected in D+ samples especially the partial D41 which is the most frequent variant phenotype that has made a clinically significant anti-D capable of causing severe HDN and neonatal death. The weak D type 4 appeared to be the most common D variant allele in Tunisians. The frequency of weak D type 4 was 0.55%. The cumulative frequency in South African blacks of the weak D type 4 is 17.2%. The cumulative frequency of weak D type 4 in white blood donors is 0.0055%.

This study improves current understanding of RHD alleles in our population. The multiplex PCR assay might be useful for screening RHD alleles especially with RHD/RHCE DNA sequence exchange mechanism and solving serological difficulty.

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