(C)ce^{s} haplotype screening in Tunisian blood donors

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Background. The *(C)ce*^{s} haplotype, mainly found in black individuals, contains two altered genes: a hybrid *RHD-CE-D*^{s} gene segregated with a *ce*^{s} allele of *RHCE* with two single nucleotide polymorphisms, c. 733C>G (p.Leu245Val) in exon 5 and c. 1006G>T (Gly336Cys) in exon 7. This haplotype could be responsible for false positive genotyping results in RhD-negative individuals and at a homozygous level lead to the loss of a high incidence antigen RH34. The aim of this study was to screen for the *(C)ce*^{s} haplotype in Tunisian blood donors, given its clinico-biological importance.

Material and methods. Blood samples were randomly collected from blood donors in the blood transfusion centre of Sousse (Tunisia). A total of 356 RhD-positive and 44 RhD-negative samples were tested for the *(C)ce*^{s} haplotype using two allele-specific primer polymerase chain reactions that detect c. 733C>G (p.Leu245Val) and c. 1006G>T (p. Gly336Cys) substitutions in exon 5 and 7 of the *RHCE* gene. In addition, the presence of the D-CE hybrid exon 3 was evaluated using a sequence-specific primer polymerase chain reaction.

Results. Among the 400 individuals only five exhibited the *(C)ce*^{s} haplotype in heterozygosity, for a frequency of 0.625%. On the basis of the allele-specific primer polymerase chain reaction results, the difference in *(C)ce*^{s} haplotype frequency was not statistically significant between RhD-positive and RhD-negative blood donors.

Discussion. These data showed the presence of the *(C)ce*^{s} haplotype at a low frequency (0.625%) compared to that among Africans in whom it is common. Nevertheless, the presence of *RHD-CE-D*^{s} in Tunisians, even at a lower frequency, should be considered in the development of a molecular genotyping strategy for Rh genes, to ensure better management of the prevention of alloimmunisation.

Keywords: *RHD* gene, *RHCE* gene, *(C)ce*^{s} haplotype, ASP-PCR, Tunisian population.

Introduction

The Rhesus (Rh) blood group system is of clinical interest because it is involved in haemolytic diseases of the newborn and haemolytic transfusion reactions. The Rh system is complex, consisting of 50 currently known antigens[1]. The most important antigens are D, C/c, and E/e. Rh antigens are carried on two proteins encoded by genes denoted *RHD* and *RHCE* in close proximity on chromosome 1. These genes are 97% identical, each has 10 exons and they encode proteins that differ by 32 to 35 amino acids.

The frequency of RhD-negative phenotype differs widely among races, being approximately 15% in Caucasians[2] and 3% to 7% in Africans[3]. The frequency in Tunisian cohorts is approximately 9% to 10%[4]. Studies have demonstrated that the molecular mechanisms in RhD-negative individuals with different ethnic backgrounds are likewise quite diverse[5]. The RhD-negative phenotype in Tunisians is mainly caused by *RHD* gene deletion[6], whereas in other ethnic groups, especially in dark-skinned (sub-Saharan) individuals who account for a relatively small proportion of the Tunisian population[7], D negativity is frequently caused by aberrant *RHD* genes, either *RHDψ* or *d(C)ce*^{s}. The molecular characteristics of this haplotype were described by Faas and colleagues[8]. The *(C)ce*^{s} haplotype contains two altered genes: an *RHD-CE-D*^{s} hybrid gene which results from a gene conversion, favoured by the opposite orientation of the *RHD* and *RHCE* genes, and a *ce*^{s} allele of the *RHCE* gene with two single nucleotide polymorphisms: c. 733C>G (p.Leu245Val) in *RHCE* exon 5 and c. 1006G>T (p. Gly336Cys) in *RHCE* exon 7 that results in a VS+V− phenotype. Thus, the *(C)ce*^{s} haplotype encodes weak C, c, weak e called e^{s} and VS antigens, whereas its does not produce D and V antigens.

The most usual and common VS/V phenotype reported so far is the VS−V− phenotype; however, two other phenotypes, related to ethnic origin, have been described recently[9]: the VS+V+ phenotype associated with the *ce*^{s} allele and the VS+V− phenotype associated with the *(C)ce*^{s} haplotype. Moreover, an unusual phenotype, VS−V^{w}+, has been reported to be associated with the *ceAR* allele resulting from an *RHCE-D-CE* gene involving RHD exon 5 with c. 48G>C, c. 712

A>G, c. 733 C>G, c. 787 A>G, c. 800 T>A, c. 916A>G nucleotide substitutions: this *RHCE* allele has been mainly described in people of African origin[10].

Pham *et al*.[11] found, in individuals of African origin, that the *(C)ce*^{s} haplotype could have two different molecular backgrounds at the level of the hybrid *RHD-CE-D*^{s}: the *(C)ce*^{s} haplotype described by Faas and colleagues[8], named "*(C)ce*^{s} haplotype type 1" and "*(C)ce*^{s} haplotype type 2". "*(C)ce*^{s} haplotype type 1" is present when the hybrid *RHD-CE-D*^{s} gene consists of exons 1 and 2, parts of 3, 8, 9, and 10 from *RHD* and the remainder of exon 3 and exons 4 through 7 from *RHCE*, whereas "*(C)ce*^{s} haplotype type 2" is present when the hybrid *RHD-CE-D*^{s} gene consists of exons 1 and 2, complete exon 3, exons 8, 9, and 10 from *RHD*; and exons 4 through 7 from *RHCE*.

Either hybrid *RHD-CE-D*^{s} allele from *(C)ce*^{s} haplotype type 1 or type 2 segregates with a *ce*^{s} allele with two nucleotide substitutions: c. 733C>G in *RHCE* exon 5 and c. 1006G>T in *RHCE* exon 7[11]. When present in the homozygous state, either the type 1 or type 2 *(C) ce*^{s} haplotype induces the absence of the high-prevalence RH34 antigen[9,11] which has a high clinical incidence in the settings of obstetrics and incompatible transfusions. Such a haplotype could be also responsible for false positive results during Rh genotyping in RhD-negative cohorts.

In this study we screened for the *(C)ce*^{s} haplotype in Tunisian blood donors, which should be considered in a reliable genotyping strategy for RhD-negative people. We analysed genomic DNA by allele-specific primer (ASP) polymerase chain reaction (PCR) for c. 733C>G and c. 1006G>T changes in *RHCE* exons 5 and 7, respectively, and studied the occurrence of *RHD-CE* hybrid exon 3 by sequence-specific (SSP)-PCR.

Materials and methods

Samples

Blood samples were collected into ethylenediaminetetraacetate (EDTA) from 400 random blood donors at the blood transfusion centre of Sousse, Tunisia.

Serological typing

For all samples, RH antigens (D: RH1, C: RH2, E: RH3, c: RH4 and e: RH5) were serologically determined using monoclonal anti-D antibody (Biomaghreb, Ariana, Tunisia). The reagent was prepared from a blend of both IgG and IgM anti-D. The IgM anti-D agglutinates with D-positive red cells except for D^{VI} and some weak D phenotype, whereas the IgG anti-D agglutinates with the D^{VI} and some weak D in an indirect antiglobulin test (IAT). Bio-Rad reagents (Loos, France) were used to test for the presence of anti-C, anti-c, anti-E and anti-e antibodies using 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). Standard serological tests were performed according to the manufacturer's instructions and the IAT was performed systematically for apparently RhD-negative results.

Molecular analysis

Genomic DNA was extracted from whole blood cells with the salting out method as described by Miller[12]. The genomic DNA of the samples studied was analysed using two ASP-PCR procedures to detect the *(C)ce*^{s} haplotype. The wild-type ASP-PCR was always performed in parallel to assess the homozygous status of the mutations. A PCR internal control was included in order to avoid false negative results. One sample known to be RH:-34 ((C)ce^{s}/(C)ce^{s}) had been used to validate the methods. Amplifications were carried out with *Taq* DNA polymerase (Invitrogen, Life technologies, São Paulo, Brazil). Primer sets are reported in Table I.

RHCE-INT4F/RH-EX5CR, RH-EX5GR primer sets were used to detect the *RHCE* C733G polymorphism in two separate PCR which were performed with 100 ng of genomic DNA in a total reaction volume of 50 µl. These reactions provide a 428-bp when the appropriate *RHCE* exon 5 sequence is present. As a control, we used a couple of primers (RH-EX7F and RHCE-EX7R) that target a non-polymorphic sequence in exon 7 of the *RHCE* gene, to amplify a 130 bp product. Reaction mixtures contained 0.1 µM of each primer, 200 µM of each dNTP, and 0.4 U of *Taq* DNA polymerase in the appropriate buffer. Amplifications were programmed on a thermal cycler (9700 GeneAmp PCR System; Applied Biosystems). Cycling conditions consisted of an initial denaturation for 5 minutes at 94 °C followed by 30 cycles carried out using the following sequence: denaturation at 94 °C for 30 seconds, primer annealing at 64 °C for 30 seconds and polymerisation at 72 °C for 30 seconds.

Table I - Oligonucleotides primers used for the three polymerase chain reactions.

Primer name	Primer direction	Sequence 5'to 3'	References
RHCE-INT4F	Forward	GCAACAGAGCAAGAGTCCA	
RH-EX5CR	Reverse	TGTCACCACACTGACTGCTA**G**	
RH-EX5GR	Reverse	TGTCACCACACTGACTGCTA**C**	
RH-EX7F	Forward	AACCCGAGTGCTGGGGATTC	
RHCE-EX7R	Reverse	ACCCACATGCCATTGCCGTTC	**13**
RH-EX7GF	Forward	ACTCCATCTTCAGCTTGCTG**G**	
RH-EX7TF	Forward	ACTCCATCTTCAGCTTGCTG**T**	
RHD-EX3F	Forward	TCGGTGCTGATCTCAGTGGA	
RHCE-EX3R	Reverse	ACTGATGACCATCCTCAGGG	
HGH F	Forward	GCCTTCCCAACCATTCCCTTA	**14**
HGH R	Reverse	TCACGGATTTCTGTTGTGTTTC	

RH-EX7GF and RH-EX7TF/RHCE-EX7R primer sets were used to detect the G1006T polymorphism: these primers amplified a 94-bp product when the appropriate *RHCE* exon 7 sequence was present. The control primers (HGH F and HGH R) amplified a 429-bp segment of a conserved region of human growth hormone. PCR amplifications were performed using the following conditions: an initial denaturation for 5 minutes at 95 °C and then 30 cycles of 95 °C for 30 seconds, 63 °C for 30 seconds and 72 °C for 30 seconds.

The presence of the *RHD-CE* hybrid exon 3 was analysed using SSP-PCR involving a forward primer (RHD-EX3F) that is specific for the 5' end of *RHD* exon 3 and a reverse primer (RHCE-EX3R) that is specific for the 3' end of *RHCE* exon 3. These primers will only amplify a *RHD-CE* hybrid exon 3 to produce a 110-bp product. The control primers (RH-EX7F and RHCE-EX7R) amplified a 130-bp product from exon 7 of *RHCE*. PCR amplification parameters were an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 30 seconds, at 65 °C for 30 seconds and at 72 °C for 45 seconds.

All PCR were terminated after extension for 10 minutes at 72 °C. The PCR products were subjected to agarose gel electrophoresis and visualised with ethidium bromide staining.

Statistical analysis

(C)ce^s haplotype screening among RhD-positive and RhD-negative donors was assessed using the Student's *t* statistical test.

Results

Serological analysis

Serological tests were performed for the 400 blood donors of whom 356 were typed RhD-positive and 44 typed RhD-negative. DCcee was found to be the most prevalent phenotype (44.38%) among RhD-positive blood donors and ddccee (68.18%) the most prevalent among RhD-negative blood donors.

Molecular biology

ASP-PCR DNA analysis of RHCE exons 5 and 7 and SSP-PCR of *RHD-CE* hybrid exon 3 were carried out in all donors to detect the c. 733C>G and c. 1006G>T polymorphisms and the *RHD-CE-D* gene; these combined nucleotides and hybrid exon 3 have been associated with the presence of *(C)ce^s* haplotype type 1.

Five samples of which one was RhD-negative with the ddCcee phenotype and four were RhD-positive including two samples with a DCcee phenotype and two others with a DCcEe phenotype exhibited, in a heterozygous state, both c. 733C>G (Figure 1) and c. 1006 G>T (Figure 2) substitutions in correlation with *RHD-CE* hybrid exon 3 (Figure 3) suggesting the presence of *(C)ce^s* haplotype type 1 with an allelic frequency of 0.625% in our studied cohort. The difference between *(C)ce^s* haplotype frequency among RhD-positive and RhD-negative blood donors was not statistically significant (Student's *t*-test value=0.45). Furthermore no *(C)ce^s* type 2 allele was found and homozygosity for the *(C)ce^s* haplotype was not observed either.

Among the other 395 donors testsed, ASP-PCR amplifications showed that 58 samples were from donors heterozygous for C/G at nucleotide 733 and 12 were from homozygous donors; no c. 1006G>T mutation was detected in any of these donors. The 325 remaining donors were negative for the two analysed nucleotide substitutions (Table II).

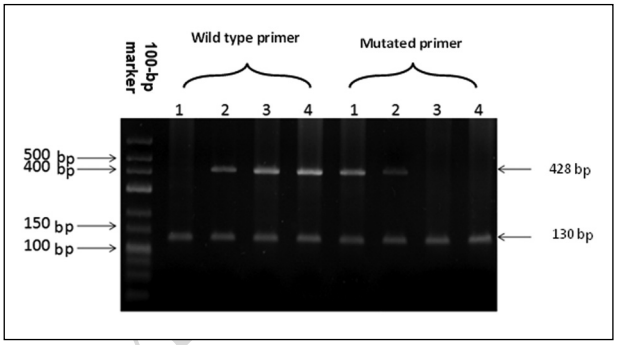

Figure 1 - ASP-PCR pattern of RHCE Exon 5 1: G733/G733 (RH: -34 phenotype) 2: C733/G733 (mutation present at heterozygous level) 3, 4: C733 /C733 (mutation absent).

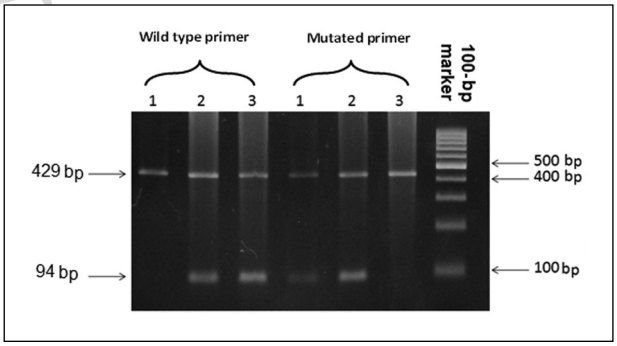

Figure 2 - ASP-PCR pattern of RHCE Exon 7 1: T1006/T1006 (RH: -34 phenotype) 2: G1006/T1006 (mutation present at heterozygous level) 3: G1006 /G1006 (mutation absent).

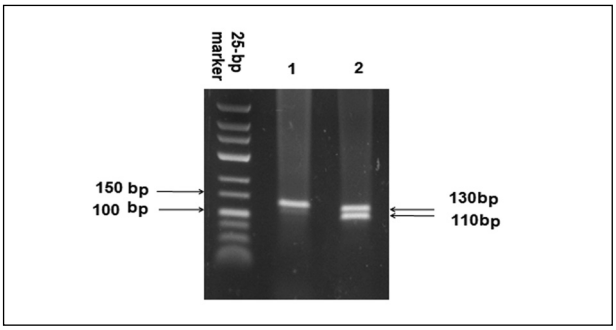

Figure 3 - Agarose gel showing results of SSP-PCR screening test for the RHD-CE hybrid Exon3 1: RHD-CE hybrid exon 3 absent 2: RHD-CE hybrid exon 3 present.

Table II - c. 733 C>G, c. 1006 G>T and *RHD-CE* exon 3 PCR results.

	C733G	G1006T	*RHD-CE* hybrid exon 3*
RhD-positive (n=356)	CC (284)	GG (284)	− (285)
	CG (59)	GG (55)	− (55)
		GT (4)	+ (4)**
	GG (12)	GG (12)	− (12)
RhD-negative (n=44)	CC (40)	GG (40)	− (40)
	CG (4)	GG (3)	− (3)
		GT (1)	+ (1)**

*: − = *RHD-CE* hybrid exon 3 absent; + = *RHD-CE* hybrid exon 3 present;
**: results suggesting the presence of the *(C)ce*^{s} haplotype in a heterozygous state.

Discussion

In this study, genomic DNA from 400 random donors (356 RhD-positive, 44 RhD-negative) was screened for the *(C)ce*^{s} haplotype, which produces a low incidence antigen (VS antigen) and in a homozygous state leads to the loss of a high incidence RH34 antigen which is of clinical interest in transfusion reactions and obstetrics essentially in inter-population unions[10]. We used PCR amplifications to detect c. 733C>G and c. 1006 G>T nucleotide substitutions in *RHCE* exons 5 and 7, respectively, and to test for the presence of *RHD-CE* hybrid exon 3.

Our study showed the presence of c. 733C>G and c. 1006 G>T substitutions in a heterozygous state in five out of the 400 cases, as well as the *RHD-CE* hybrid exon 3 in favour of presence of the *(C)ce*^{s} haplotype in heterozygosity. This haplotype occurred in 0.625% of the Tunisians, which is much lower than the frequency reported in Africans in Mali[15]. The presence of this haplotype in Tunisians could be linked to the ethnic heterogeneity of Tunisian population, which is a mixture consisting mainly of Berbers -the autochthonous population- and Arabs, with a relatively small sub-Saharan African contribution that could be at the origin of the individuals carrying the *(C)ce*^{s} haplotype[7].

Four out of the five cases were RhD-positive with DCcee and DCcEe phenotypes and, respectively, eventual *DCe/d(C)ce*^{s} and DcE/*d(C)ce*^{s} genotypes. The fifth individual was RhD-negative with a ddCcee phenotype and *d(C)ce*^{s}*/dce* genotype; the dce haplotype could have resulted from an *RHD* gene deletion or another RhD-negative allele such as *RHDψ* or an *RHD-CE-D* hybrid gene. These five cases exhibited the classical *(C)ce*^{s} haplotype as described by Faas and co-workers and named *(C)ce*^{s} type 1 by Pham and colleagues[8,11].

The five donors carrying the *(C)ce*^{s} haplotype could be presumed to present the VS+V- phenotype since VS expression resulted from a c. 733C>G substitution that predicts the p. Leu245Val in the RHCE protein. Conversely, absence of V in the *(C)ce*^{s} haplotype was suggested to be linked to the presence of the c. 1006 G>T substitution predicting Cys336 in the protein[13]. In fact, two studies have shown an association between VS and RHCE G733 encoding Val245. Steers *et al.* identified the presence of the G733 mutation in 55 VS+ samples analysed whereas 180 VS- samples showed a C733[16]. Faas *et al.* demonstrated that 43 VS+ donors were homozygous or heterozygous for G733[8]. Nevertheless, the c. 733C>G and c. 1006 G>T ASP-PCR tests may not be sufficient to deduce VS and V antigenicity and only serological typing could allow a definite conclusion to be drawn about the expression of these antigens.

Fifty-eight donors were heterozygous for c. 733 C>G and 12 others were homozygous without c. 1006 G>T substitution; all of these donors appear to have the ce^s allele. The probable genotypes of these donors are summarised in Table III.

Table III - Serological phenotype and probable genotype of 70 blood donors.

Number	Phenotype	Nucleotide 733	Nucleotide 1006	Probable genotype
58	DCcee (31)	C/G	G/G	DCe/ce^s
	Dccee (15)	C/G	G/G	Dce/ce^s
	DccEe (9)	C/G	G/G	DcE/ce^s
	ddccee (3)	C/G	G/G	dce/ce^s
12	Dccee (12)	G/G	G/G	Dce^s/ce^s

It was well established in previous studies that the *(C)ce*^{s} haplotype when present in a homozygous state results in the RH:-34 phenotype[11] which is of marked clinical relevance in transfusion settings and obstetrics[10,17]. Given the presence of this haplotype in the cohort we studied, in a heterozygous state with a frequency of 0.625%, it can be presumed that the RH:-34 phenotype exists in the Tunisian population.

Thus, in Tunisia the *RHD-CE-D*^{s} allele should be considered in the development of a molecular strategy for *RHD* genotyping of RhD-negative people by targeting at least three *RHD*-specific sequences, in order to limit false positive results, particularly in foetal RhD genotyping.

The Authors declare no conflicts of interest.

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