Insights into RHCE Molecular Analysis in Samples with Partial D Variants: the Experience of Western France

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Introduction

Systematic genotyping of blood donor samples is currently technically feasible by high-throughput platforms [1–7] or next-generation sequencing in a near future [8, 9], although it remains to definitely confirm whether the clinical benefit significantly over-passes the economic cost. Serological analyses, which remain the gold standard, are known to be potent and robust to investigate blood group status and are sufficient to achieve this aim most of the time, unless specific antisera are not available. Therefore, an extensive, systematic genotyping of major blood group genes (for example RHD and RHCE in Rh; DARC in Duffy; SLC14AJ in Kidd; KEL in Kell; GYP A and GYP B in MNS) in all donors may not be required from a strict transfusion point of view. This statement is however challenging regarding multi-transfused patients suffering from hemoglobinopathies, such as sickle cell disease (SCD). These patients are treated by transfusions on a regular basis with red blood cell (RBC) units from different donors that are practically not ethnically matched. Because this population of African descent is prone to express partial antigens (e.g., partial e) while others commonly found in Caucasians are not expressed (e.g., Fy), transfused RBC units may induce allo-immunization, thus complicating subsequent cross-matching.

In that context, extended genotyping in selected subsets of samples may be particularly worthwhile. The Rh system is extremely complex, not only in terms of molecular genetics but also because of the 54 antigens reported so far, some of them being of major clinical significance [10]. For years it has been demonstrated that some RHD variants, particularly those of the three African clusters [11], segregate with RHCE variants, which result in the expression of partial/rare antigens [12–19]. But it is currently unknown to

Keywords
Antigens · Blood group · Genotyping · RHCE · RHD · Variant alleles

Summary
Background: Although systematic blood group genotyping of patients/donors is virtually possible, serological studies remain the gold standard to identify samples of clinical interest that may be further genotyped. In this context, we sought to identify variant D alleles that are likely to be clinically relevant in terms of other Rh antigens in a subset of population genotyped in Western France.

Methods: Samples presenting with the RHD*weak D type 4.2.2 allele (n = 47) were selected for the study. RHCE exons 1–7 were directly sequenced, and expression of Rh antigens was predicted on the basis of the molecular data.

Results: Of the 47 samples tested, 19 (40.4%) were predicted to be of potential clinical interest. Moreover, we could show that selecting the samples to be genotyped by the nature of their variant D allele (i.e., RHD*weak D type 4.2.2 allele) rather than by their Duffy-null status appears to increase significantly the likelihood of identifying clinically relevant individuals for Rh status.

Conclusion: On the basis of our findings we suggest that all individuals genotyped as weak D type 4.2.2 should be systematically screened for RHCE variants by molecular analysis on a routine basis.

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what extent expression of various antigens carried by the CE allele is linked to the expression of variant D alleles. On the basis of that statement, we sought to identify variant D carriers that are likely to be clinically relevant in terms of the other Rh antigens in the population of blood donors and patients in Western France. To this aim the RHCE gene was genotyped in a subset of 47 samples carrying the RH*D*weak D type 4.2.2 allele. Phenotypes were predicted on the basis of the sequencing data. Here we report the results of that study.

Material and Methods

DNA Samples

Among the 2,000 DNA samples from donors and patients collected and addressed to our laboratory by the local sites of the French Blood Center (Etablissement Français du Sang, France) in Western France from 2003 to 2013, DNA samples characterized locally by routine molecular methods [20–22] as RH*D*weak D type 4.2.2 were selected for the study, for a total of 47 unrelated DNA samples characterized locally by routine molecular methods [20–22] as RH*D*weak D type 4.2.2 were selected for the study. For simplicity, samples presenting with heterozygous sequencing profiles in the RHD gene were excluded from the analysis, implying that only hemizygous and homozygous D variants were further studied.

Molecular Analyses and Phenotype Prediction

Exons 1 up to 7 of the RHCE gene (NM_002036.3) were specifically PCR-amplified and directly sequenced in all DNA samples with primers 5'-TGTAGTCCCAAC -CAGCCAAATC-3' and 5'-untranslated region (UTR) of the DARC gene (NM_0020485.4) were specifically PCR-amplified and directly sequenced by following previously published conditions [23]. RHCE exon 6 was alternatively PCR-amplified by using forward primer rb25 [24] prior to direct sequencing. To simplify the molecular testings and because a limited number of RHCE alleles including variants within exons 8, 9, or 10 have been reported so far (ISBT Blood Group Allele Terminology: www.isbtweb.org/fileadmin/user_upload/WP_on_Red_Cell_Immunogenetics_and/004_RHCE_alleles_v2.0_110914.pdf), the three last exons of the gene were not investigated.

RH*D*weak D type 4.2.2 was considered as significant.

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alleles on a RHCE*ce background (fig. 1), including the recently reported RHCE*ce(c.48C, c.105T) allele [25], which is reminiscent to the RHCE*ce.01 allele with an additional silent c.105C>T variant, and a yet unreported RHCE*ce(c.712A, c.733G, c.787G, c.800A, c.916G) allele, which only differs from the RHCE*ce.04 allele by the c.48G>C transversion that is not found here. QMPSF analysis of the 47 samples genotyped as weak D type 4.2.2 exhibited aberrant results in five DNAs (data not shown), which is a typical feature of degraded genomic DNA [22]. Then RHD zygosity could not be investigated in these samples. Of the 42 remaining samples, four were found to carry two copies of the RHD*weak D type 4.2.2 allele, while the 38 others carry one variant D copy (data not shown). In terms of RHCE exon numbering, five different profiles affecting some or all exons 1, 5, 6, and 8 were found (table 1, fig. 2). Patterns with altered numbering of exons 1, 5, and 6, for a total of 36 samples, are linked to the respective base substitutions carried by the RHCE*ce.04 allele that directly impair QMPSF primer annealing. Only two samples display alternative profiles (table 1): in the RHCE*ce/RHCE*ce.05 sample, one copy of markers 1C and 5 are found, while the RHCE*ce.20.01/RHCE*ce.20.02 sample exhibits one copy of marker 1C. As observed before, exon 8 marker, which targets a genomic sequence upstream exon 8, may vary between 0 and 4 in both genes depending on its origin [22].

Initially discrepant results were found between QMPSF and direct sequencing in eight samples in RHCE exon 6. Indeed, while QMPSF data indicated one copy of this marker with a forward primer targeting the c.916A, RHCE-specific position, direct sequencing suggested a homozygous G nucleotide at that position. RHCE exon 6 was then PCR-amplified by using an alternative forward primer (i.e., rb25, see 'Material and Methods') and then sequenced, resulting in a heterozygous A/G pattern at position c.916, as expected. Overall the QMPSF profiles are in full accordance with the sequencing data.

Finally a total of nine genotypes could be predicted (table 2). As reported in the literature, RHCE*ce.04 (also known as ccar; 45/47 individuals, 95.7 %) and RHCE*ce.05 (also known as ceEK; 1/47, 2.1 %) were found to be linked to the RHD*weak D type 4.2.2 allele [15, 26–29], while only one DNA does not carry either allele. Further serological investigation will be required to characterize more precisely antigen expression resulting from these allele combina-
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Table 2. RHCE allele, phenotype prediction and Fy(a–b–) status in 47 weak D type 4.2.2 samples

<table>
<thead>
<tr>
<th>RHCE genotype prediction</th>
<th>Occurrence</th>
<th>Potential clinical interest</th>
<th>Fy(a–b–)</th>
<th>Fy(a–b–) + potential clinical interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>'ce.04/1'ce</td>
<td>22 (46.8%)</td>
<td>no</td>
<td>8 (17.0%)</td>
<td>N/A</td>
</tr>
<tr>
<td>'ce.04/1'ce.20.01</td>
<td>8 (17.0%)</td>
<td>yes</td>
<td>8 (17.0%)</td>
<td>8 (17.0%)</td>
</tr>
<tr>
<td>'ce.04/1'ce.05</td>
<td>6 (12.8%)</td>
<td>yes</td>
<td>4 (8.5%)</td>
<td>4 (8.5%)</td>
</tr>
<tr>
<td>'ce.04/1'ce.04</td>
<td>3 (6.4%)</td>
<td>yes</td>
<td>3 (6.4%)</td>
<td>3 (6.4%)</td>
</tr>
<tr>
<td>'ce.04/1'ce.01</td>
<td>3 (6.4%)</td>
<td>no</td>
<td>3 (6.4%)</td>
<td>N/A</td>
</tr>
<tr>
<td>'ce.04/1'ce.(c.48K, c.105T)</td>
<td>2 (4.3%)</td>
<td>no</td>
<td>2 (4.3%)</td>
<td>N/A</td>
</tr>
<tr>
<td>'ce.04/1'ce.(c.712A, c.735G, c.787G, c.809A, c.916G)</td>
<td>1 (2.1%)</td>
<td>yes</td>
<td>1 (2.1%)</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>'ce.05/1'ce</td>
<td>1 (2.1%)</td>
<td>no</td>
<td>1 (2.1%)</td>
<td>N/A</td>
</tr>
<tr>
<td>'ce.20.01/1'ce.02</td>
<td>1 (2.1%)</td>
<td>yes</td>
<td>1 (2.1%)</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>47 (100.0%)</td>
<td>19 (40.4%)</td>
<td>31 (66.0%)</td>
<td>17 (36.2%)</td>
</tr>
</tbody>
</table>

N/A = Not applicable.

aHomozygous RHD*weak D type 4.2.2 samples.
bThe c.105C>T substitution is a silent variant.
cto be confirmed by further serological analyses.

tions, especially regarding partial c and partial e. But on the basis of the molecular data, 19 samples are predicted to be potentially of clinical interest (table 1).

Recently, Kappler-Gratias et al. [30] reported a study in which systematic RH genotyping was carried out in 316 blood donors of African origin selected on the basis of the Fy(a–b–) phenotype that is typical of sub-Saharan populations [31]. Of the 31 donors carrying one or two variant D alleles of various molecular structures resulting in a partial D phenotype, two samples (i.e., 1 RHD*weak D type 4.2.2 and 1 RHD*DIIIa; 6.5%) were reported with a predicted, rare Rh phenotype. Because we did not have access to the Fy phenotype in our cohort, we genotyped the c.-67 nucleotide position of the DARC gene by direct sequencing, which is characterized by a T>C substitution at the homozygous state in Fy(a–b–) samples [32]. 31 out of 47 samples (66.0%) were predicted to be Fy(a–b–) (table 2), which is in the range of what reported before (63.0%; $\chi^2 = 0.12; p = 0.7276$) [33] and confirms the African origin of our cohort. Of this subset, 17 were potentially of clinical interest (table 2), which is significantly more than what was observed by Kappler-Gratias et al. [30] (6.5% vs. 54.8% in this study; $\chi^2 = 17.07; p = 0.000036$). This latter result suggests that selecting samples to be genotyped within the RHCE gene on the basis of their variant D allele rather than their African origin may contribute to optimize the probability to identify rare, clinically relevant Rh phenotypes.

**Discussion**

Systematic molecular genotyping of blood group genes has been made technically feasible by the tremendous improvements of high-throughput technology over the past 10 years. Although the clinical benefit of mass-scale genotyping has been reported [34, 35], the economic issue remains a major hurdle to routinely implement such a strategy. To gain insights into the accurate identification of samples of potential clinical interest that may deserve further genotyping, we focused our study on a subset of samples selected on the basis of preliminary molecular criteria and took advantage of our local DNA bank. Our analysis stressed on the RH genes, which are known to express more than 50 antigens [10]. Because many variant RHCE alleles have been reported in people of African origin, we sought to evaluate the relevance of genotyping that gene in 47 samples addressed for routine RHD molecular analysis and presenting with a RHD*weak D type 4.2.2 variant at either the hemizygous or the homozygous state. The respective Rh phenotype was subsequently deduced from molecular data. It is worth mentioning that i) segregation of the variant D allele with the RHCE allele could not be investigated and ii) this prediction is exclusively based on the genotyping results in line with the data found in the literature, suggesting that further serological investigations are mandatory to confirm our predictions. From another point of view it is also important to note that in the context of systematic molecular mass-scale screening of all donors, i) haplotyping would not be carried out and ii) assignment of blood group status would be preliminary based on these molecular data, then suggesting that our prospective study falls into that latter area.

Nine different allele combinations could be deduced in the 47 samples, among which 19 (40.4%) (table 2) were assumed to be clinically relevant in terms of Rh status. While the clinical significance of anti-V and anti-VS has remained mild if any so far [10], expression of partial c and partial e as well as lack of expression of the high-frequency antigens Hr and Hr B together with hrS and hr B , respectively, is a critical issue [15, 28, 29, 36–40], particularly in a transfusion context in patients that are prone to be transfused on a regular basis. Of note, it must be mentioned that anti-c has been reported in individuals carrying RHCE*ce.04 [28, 29], this latter allele being importantly found in 45/47 samples.

As expected, it is interesting to underline that the QMPSF assay [22] easily provided clues about the nature of the RHCE*ce.04 allele by displaying a typical pattern. Indeed, the hybrid CE-D exon 5 is not amplified by the QMPSF primers and the c.916A>G transition

![Image](https://example.com/image.png)


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found in this allele impairs the specific primer annealing in exon 6, resulting in 1 or 0 copy calculated for both exons in compound heterozygous or homozygous RHCE*ce04 samples. It is also worthwhile to mention that, if this latter test had not been performed, the c.916 position in RHCE exon 6 would have been incorrectly genotyped as homozygous G/G instead of heterozygous A/G in 8/47 samples. Once again this example highlights the complexity of the molecular genetics of the RH genes, because of the high degree of similarity and the multiple gene rearrangements occurring in both coding and non-coding regions, and more specifically the critical challenge to genotype accurately the RHCE gene.

A recent paper reported an important study of systematic RH genotyping in a cohort of French donors of African origin [30]. The authors interestingly concluded that specific mutations defining RH and/or RHCE variant alleles, as well as D-CE haplotype inheritance, should be taken into consideration for extended molecular analysis. Our results are definitely in favor of that statement. Furthermore, although most of the RHCE variant alleles are carried by people of African ancestry [39], we showed that selecting samples of interest on the basis of their variant D allele for subsequent RHCE genotyping would be more appropriate than by their origin (i.e., Fy(a–b–) phenotype) (table 2).

In conclusion we have provided molecular data suggesting that both donors and patients genotyped as weak D type 4.2.2 should be systematically screened for RHCE variants by molecular analysis on a routine basis. This strategy may help to prevent Rh alloimmunization in a transfusion context by reinforcing data to favor genetic matching, especially by considering that these individuals are particularly prone to be involved in SCD management. It will be mandatory to confirm and complete these preliminary results by serological studies and/or clinical data to define general guidelines for blood group typing as well as to carry out such studies with variants D samples of other clusters.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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