Systematic *RH* genotyping and variant identification in French donors of African origin

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Background. *RH* molecular analysis has enabled the documentation of numerous variants of *RHD* and *RHCE* alleles, especially in individuals of African origin. The aim of the present study was to determine the type and frequency of D and/or RhCE variants among blood donors of African origin in France, by performing a systematic *RH* molecular analysis, in order to evaluate the implications for blood transfusion of patients of African origin.

Materials and methods. Samples from 316 African blood donors, whose origin was established by their Fy(a−b−) phenotype, were first analysed using the RHD and RHCE BeadChips Kit (BioArray Solutions, Immucor, Warren, NJ, USA). Sequencing was performed when necessary.

Results. *RHD* molecular analysis showed that 26.2% of donors had a variant *RHD* allele. It allowed the prediction of a partial D in 11% of cases. *RHCE* molecular analysis showed that 14.2% of donors had a variant *RHCE* allele or *RH* [R^N or *(C)ce*^{s}] haplotype. A rare Rh phenotype associated with the loss of a high-prevalence antigen or partial RhCE antigens were predicted from *RHCE* molecular analysis in 1 (0.3%) and 17 (5%) cases, respectively.

Discussion. Systematic *RHD* and *RHCE* molecular analysis performed in blood donors of African origin provides transfusion-relevant information for individuals of African origin because of the frequency of variant *RH* alleles. *RH* molecular analysis may improve transfusion therapy of patients by allowing better donor and recipient matching, based not only on phenotypically matched red blood cell units, but also on units that are genetically matched with regards to RhCE variants.

Keywords: Rh blood group system, Rh variants, molecular analysis, DNA testing, blood donors.

Introduction

The Rh blood group system, involved in alloimmune responses after blood transfusion and in haemolytic disease of the newborn, is of great clinical importance[1]. The system comprises more than 50 antigens referenced by the International Society of Blood Transfusion (http://www.isbt-web.org)[2]. The most common ones are D (RH1), C (RH2), E (RH3), c (RH4), and e (RH5). The antigens of the Rh system are encoded by two homologous genes, the *RHD* gene encoding the D protein, and the *RHCE* gene encoding the protein carrying the C/c and E/e antigens. *RHCE* has four main alleles encoding the Ce, CE, ce and cE antigen combinations[3,4]. *RHD* and *RHCE* genes, each composed of ten exons, represent a cluster of genes[5-10]. Their respective alleles segregate as haplotypes, the frequencies of which vary according to ethnic group. The *RH* genes are a source of significant diversity favoured by the opposite orientation of *RHD* and *RHCE* genes. Some variant Rh phenotypes are caused by exchange of genetic material between the two genes, resulting in hybrid *RH* genes. Others result from missense mutations. The Rh variants can weaken expression of the common antigens, produce partial antigens, generate low-prevalence antigens, and result in absence of a high-prevalence antigen[11].

The D antigen is one of the most immunogenic blood group antigens. D variants may be differentiated into weak D and partial D. The weak D phenotype first described in 1946 was related to red blood cells reacting in an atypical manner with anti-D[12]. Nowadays, a weak D red blood cell can be defined as a red blood cells giving a weaker reaction than red blood cells of the same Rh phenotype as reference, according to a defined anti-D reagent and a defined technique. Partial D phenotypes are characterised by loss of epitopes. Patients expressing a partial D have the potential to produce alloanti-D against the part of D that they lack. More recently, D variants have been classified at the molecular level. Based on *RHD* sequence variations, mutations changing the amino acid sequence predicted to be in the membrane-spanning or intracellular regions of the RhD protein were related to a feature of weak D, whereas mutations changing the amino acid sequence predicted to be in the extracellular regions were related

to a feature of partial D[13]. On the one hand, weak D are the most frequent type of D variants found in Caucasian individuals[14]. On the other hand, partial D are the most frequent type of D variants found in individuals of African origin[14,15].

RhCE variants whose carriers may develop anti-Rh antibodies of clinical significance often demonstrate ethnic variability[16]. Many variant *RHCE*ce* alleles or *RH* haplotypes have been described in individuals of African origin: the R^N haplotype (*RHD* gene paired with a hybrid *RHCE-D-CE* gene involving either *RHD* exon 4 alone, or part of *RHD* exon 3 and exon 4)[17]; the *(C)*ce^s haplotype (a hybrid *RHD-CE-*D^s gene paired with an altered ce^s allele of *RHCE*)[18,19]; the *RHCE**ce^s*1006* allele (733C>G, 1006G>T)[20,21]; the *RHCE*ceAR* allele (48G>C, 712A>G, 733C>G, 787A>G, 800T>A, 916A>G)[22-24]; and the *RHCE*ceMO* allele (48G>C, 667G>T)[25]. We recently found that the most frequent variant *RHCE*ce* alleles or *RH* haplotypes in individuals of African origin were the R^N haplotype, the *RHCE*ceMO* allele, the *(C)*ce^s haplotype/ce^s*1006* allele, and the *RHCE*ceAR* allele when samples referred to our laboratory for altered expression of RhCE antigens and/or production of anti-RhCE in the presence of the corresponding antigen were examined[21].

The aim of the present study was to determine the type and frequency of D and/or RhCE variants among blood donors of African origin in France, by performing a systematic *RH* molecular analysis. The African origin of the blood donors was established by their Fy(a−b−) phenotype, since the ethnic origin of individuals cannot be stated or documented in donor information in France. This work was performed in order to evaluate the implications for blood transfusion of patients of African origin, such as patients with sickle cell disease needing frequent transfusion therapy.

Materials and methods

Samples

A total of 316 samples from blood donors of African origin were analysed. The African origin of the blood donors was established from their Fy(a−b−) phenotype. The male/female representation was equal: 166 males (53%) and 150 females (47%). The blood donations were made in accordance with French regulations. EDTA blood (15 mL) and serum (15 mL) were drawn from these individuals for laboratory tests to determine their suitability as donors and for systematic molecular biology analyses of *RHD* and *RHCE*. The consent of all donors was obtained before donation.

Serological typing

Donors' red blood cells were phenotyped for D, C, E, c and e with two commercial monoclonal reagents, IgG and IgM for D antigen and IgM for C, E, c and e antigens (Diagast, Loos, France) on Beckman Coulter PK 7200 and PK 7300 automated systems (Beckman Coulter, Brea, CA, USA).

Fy phenotyping of the samples was performed on the Techno TwinStation (BioRad, Hercules, CA, USA) automated analyser for gel cards with anti-Fy^a and anti-Fy^b reagents (BioRad, Hercules, CA, USA).

Molecular analysis

DNA preparation

Genomic DNA was extracted from aliquots of EDTA whole blood either on the QIAsymphony SP robot (Qiagen, Hilden, Germany) using the QIAsymphony DNA Midi Kit (Qiagen, Hilden, Germany) or on the MagNA Pure Compact instrument (Roche Molecular Biochemicals, Mannheim, Germany) using a MagNA Pure Compact Tip Tray Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

RHD *and* RHCE *gene analysis*

All samples were analysed using RHD and RHCE BeadChips Kits (BioArray Solutions, Immucor, Warren, NJ, USA).

The RHD BeadChips Kit includes 36 genetic markers. According to the instructions for users, the RHD BeadChips Kit allows detection of the following weak D, partial D and D negative variant alleles (i) *RHD*weak D type, 1,2,3,4.0,4.1,4.2/DAR, 5,11,15,17*; (ii) partial D variant alleles, *RHD*DIIIa* (DIII type 5), *RHD*DIII type 4,6*; *RHD*DIIIc*; *RHD*DIVa*; *RHD*DIVa-2*; *RHD*DIV type 3,4,5*; *RHD*DIVb*; *RHD*DV type 1,2,4,6,7,8,9; RHD*DBS-0; RHD*DHK; RHD*DVI type 1,2,3,4; RHD* DNB; RHD*DHMi; RHD*DUC-2; RHD*DAU 1,2,3,4,5; RHD*DBT 1,2; RHD*DCS 1, 2; RHD*DOL; RHD*DOL-3; RHD*DFR; RHD*DFR-2; RHD*DTO; RHD*DBS-0,1,2*; and (iii) ten D negative variants: *RHDψ; RHD*D(W16X); RHD-CE(3-7)-D; RHD-CE(4-7)-D; CcdeS; RHD-CE(3-9)-D; RHCE(1-3)-D(4-10); DEL RHD(1227G>A); DEL RHD(IVS3+1G>A); DEL RHD(M295I)*.

The RHCE BeadChips Kit enables detection of C (RH2), c (RH4), E (RH3), e (RH5), CW (RH8), CX (RH9), V (RH10), VS (RH20), and Crawford (RH43) antigens. Mutations, polymorphisms and genetic conversion can also be detected with this kit. The following *RHCE* variant alleles/haplotypes can be identified: *RHCE*ceRT, RHCE*ceAR, RHCE*ceMO, RHCE*ceRA, RHCE*CeVG, RHCE*ceEK, RHCE*ceBI, RHCE*CeMA, RHCE*ceSL, RHCE*CeVA, RHCE*ceTI, RHCE*ceFV, RHCE*DHAR, RHCE*E type I, RHCE*E type III, RHCE*E type IV, RHCE*EKH, RHCE**ce^S,

*(C)ce^{S}, RHCE*ce^{S} (340), RHCE*ce^{S} (748),* and *RHCE*16C*.

The assay was performed according to the manufacturer's instructions. Briefly, a specimen of 8 µL of DNA was added to a proprietary master mix to produce a 25 µL multiplexed polymerase chain reaction (PCR) amplification which was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA), in a "single-tube" format, using a set of primers designed to generate various amplicons containing the designated polymorphic sites of interest. The single-strand amplicons produced by post-amplification processing were combined with elongation mixture (BioArray Solutions) and were placed onto each BeadChip carrier. An image of the fluorescence pattern was taken automatically with an Array Imaging System for each chip. The web-based software BASIS analysed the image and automatically generated genotype reports.

Variants *RHD* or *RHCE* alleles found when using the RHD and RHCE BeadChips were confirmed by performing allele-specific PCR or *RHD* and *RHCE* sequencing. PCR exon amplification was performed on genomic DNA for sequence analysis. Primer sequences and PCR conditions are presented in Supplementary Table I. Primer sets used for amplification of *RHD* exons 2, 3, 6, 7, 8, 9, and 10 have been described by Legler and colleagues[26]. Primer sets used for amplification of *RHD* exons 1 and 4 to 5, *RHCE* exons 1, 3, 4 to 5, 6, and 7 have been previously described (supporting information is available in Supplementary Table II)[27]. *RHD* exons 2, 3, 6, 7, 8, 9, and 10 PCR procedures were performed in a thermal cycler on 100 ng of genomic DNA in a total reaction volume of 50 µL. Reaction mixtures contained 10 µmol/L of each primer, 200 µmol/L of each dNTP (Amersham Biosciences, Buckinghamshire, UK), and 2.5 U of *Taq* DNA polymerase (Gold, Applied Biosystems) in the appropriate buffer supplemented with 1.5 mmol/L of $MgCl_2$. *RHD* exons 1 and 4 to 5 PCR and *RHCE* exons 1, 4 to 5, 6, and 7 PCR were performed in a thermal cycler on 100 ng of genomic DNA in a total reaction volume of 50 µL. Reaction mixtures contained 10 µmol/L of each primer, 200 µmol/L of each dNTP (Amersham Biosciences), and 1 µL of *Taq* DNA polymerase (Advantage 2 polymerase mix, Clontech Laboratories, Mountain View, CA, USA) in the appropriate buffer. PCR products were purified (Microcon YM-50, Millipore Corp., Billerica, MA, USA) and cycle sequenced using big dye terminator chemistry (ABI-Prism BigDye Terminator v1.1 cycle sequencing kits, Applied Biosystems). Sequences were analysed on an automated fluorescence-based genetic analyser (ABI Prism 3130, Applied Biosystems).

The presence of the R^{N} haplotype (normal *RHD* gene paired with a *Ce-D(4)-Ce* hybrid *RHCE* gene) was systematically determined by performing an allele-specific PCR on samples from C+ donors. The *RHCE* allele-specific primer (ASP) amplification has already been described[27]. The ASP amplification analysing the genetic conversions D-Ce(3-8)-D (455A>C) and Ce-D(4)-Ce (R^{N} haplotype) were performed using primers specific to the *GH1* gene as PCR internal control, in order to avoid false-negative results. The primers and PCR conditions of ASP PCR are presented in Supplementary Table I. PCR were performed in a thermocycler (Model 9700 or 2700, Applied Biosystems, Forster City, CA, USA).

Results

The Rh phenotypes of the 316 African blood donors are shown in Table I. The D, C, E, c, and e antigens were present in 88.6%, 26.6%, 10.1%, 93.6% and 98.7% of the donors, respectively. None of these donors had weakened expression of Rh antigens, while controls gave reactions ranging from a normal 4+ reaction to a negative reaction. Antibody testing was negative in all blood donors.

Table I - Rh phenotype of the 316 blood donors of African origin.

Phenotype	ISBT phenotype	Number of samples
D+CñEñc+e+	RH:1,ñ2,ñ3,4,5	172 (54.4%)
D+C+Eñc+e+	RH:1,2,ñ3,4,5	56 (17.7%)
DñCñEñc+e+	RH:ñ1,ñ2,ñ3,4,5	31 (9.8%)
D+CñE+c+e+	RH:1,ñ2,3,4,5	25 (7.9%)
D+C+Eñcñe+	RH:1,2,ñ3,ñ4,5	20 (6.3%)
DñC+Eñc+e+	RH:ñ1,2,ñ3,4,5	5 (1.6%)
D+CñE+c+eñ	RH:1,ñ2,3,4,ñ5	4 (1.2%)
D+C+E+c+e+	RH:1,2,3,4,5	3 (0.9%)

Variant *RHD* alleles

Systematic molecular analysis of the *RHD* gene showed that 83 out of the 316 donors (26.2%) had a variant *RHD* allele. These variant *RHD* alleles were the *RHD*Psi, RHD*DAU-3, RHD*weak D type 4.0*, *RHD*DIII type 5, RHD*weak D type 4.2.2, RHD*DAU-0, RHD*DIVa–2, RHD*DAU-5, RHD*DFV, RHD*DV type 1*, and *RHD*DOL-2* alleles and occurred at frequencies of 10.7%, 4.1%, 3.8%, 2.8%, 2.2%, 2.2%, 1.9%, 1.9%, 0.6%, 0.3%, and 0.3%, respectively.

The distribution of the variant *RHD* alleles found among the 280 D+ donors in this study is reported in Table II. The D+ phenotype had to be changed into a partial D phenotype, predicted from *RHD* molecular analysis, in 31 out of these 280 donors (11%), according to the Rhesus base classification (http://www.uni-ulm.de/%7Efwagner/RH/RB/). These 31 donors were found to have one

Table II - Variant *RHD* alleles identified among D+ blood donors of African origin.

Variant *RHD* alleles*	Number	D phenotype predicted from *RHD* molecular analysis
Variant *RHD* allele present in the homozygous state or as a single *RHD* allele		
DAU-3	5	Partial D
Weak D type 4.0	2	Partial D
Weak D type 4.2.2	3	Partial D
DIVa-2	2	Partial D
DFV	1	Partial D
DIII type5	2	Partial D
DOL-2	1	Partial D
Variant *RHD* alleles present in compound heterozygosity		
DAU-3 + PSI	2	Partial D
Weak D type 4.0 + PSI	2	Partial D
DIVa-2 + PSI	1	Partial D
DIIItype5 + PSI	1	Partial D
DV type 1 + PSI	1	Partial D
DAU-5 + PSI	1	Partial D
DAU-3 + DAU-0	5	Partial D*
Weak D type 4.0 + DAU-5	1	Partial D
Weak D type 4.0 + DAU-0.1[†]	1	Partial D
Variant *RHD* alleles present in the heterozygous state		
Normal *RHD* allele + *PSI*	18	D
Normal *RHD* allele + *DIII type5*	6	D
Normal *RHD* allele + *Weak D type 4.0*	6	D
Normal *RHD* allele + *Weak D type 4.2.2*	4	D
Normal *RHD* allele + *DAU-5*	4	D
Normal *RHD* allele + *DIVa-2*	3	D
Normal *RHD* allele + *DAU-3*	1	D
Normal *RHD* allele +*DFV*	1	D
Normal *RHD* allele + *DAU-0.1*	1	D

*D antigen was considered as partial when it was encoded by two variant *RHD* alleles present in compound heterozygosity; [0]According to the Rhesus base classification (http://www.uni-ulm.de/%7Efwagner/RH/RB/).

variant *RHD* allele present in the homozygous state or as a single *RHD* allele, one variant *RHD* allele associated with the silent *Psi* allele, or two different variant *RHD* alleles in compound heterozygosity with each other in 17 donors, 7 donors, and 7 donors, respectively (Table II). Indeed, donors displaying two different variant *RHD* alleles, each allele encoding a partial D, were considered as expressing a partial D, even though no description of alloanti-D has been published.

Among the 36 D− donors, the D− phenotype was associated with different molecular backgrounds. A complete *RHD* deletion, deduced from the absence of *RHD* exon amplification, occurred in 24 samples (67%). The D− phenotype was associated with the silent *Psi* allele in seven donors, with the *(C)ce*^{s} type 1 haplotype in three donors, and with the combination of the silent *Psi* allele with the *(C)ce*^{s} type 1 haplotype in one donor. Finally, a Dñ phenotype was found to be associated with a novel *RHD* variant allele in one donor. This *RHD variant* allele, characterised by DNA sequencing because of a discordant Dñ phenotype/normal *RHD* genotype when using the RHD BeadChips Kit, displayed a 952C>T mutation in exon 7, resulting in a stop codon.

Variant *RHCE* alleles or *RH* haplotypes

In this study, the term "variant *RH* haplotypes" referred to the R^{N} haplotype or the *(C)ce*^{s} haplotype (also named r^{s}).

The systematic molecular analysis of the *RHCE* gene showed that 46 out of 316 donors (14.5%) had a variant *RHCE* allele or a variant *RH* haplotype [R^{N} or *(C)ce*^{s} haplotype]. The variant *RH* haplotypes [R^{N} or *(C)ce*^{s} haplotype] were characterised and/or confirmed by ASP-PCR. The variant *RHCE* alleles or variant *RH* haplotypes were the *(C)ce*^{S} *type1* haplotype, the *RHCE*ceTI* allele, the *RHCE*ceMO* allele, the *RHCE*ceAR* allele, the *RHCE*ce*^{s}*1006* allele, the *RHCE*ceEK* allele, the *RHCE*ce*^{s}*340* allele, the *RHCE*ceBI* allele, the R^{N} haplotype, and the *RHCE*cE916* allele: their frequencies were 3.2%, 3.2%, 2.8%, 1.9%, 1.6%, 1.3%, 0.9%, 0.3%, 0.3% and 0.3%, respectively. The *RHCE*ce*^{s} allele was found to be present in the homozygous state or in the heterozygous state in 19 (6%) and 90 (28.5%) of the 316 donors, respectively.

No variant *RHCE*ce* allele or variant *RH* haplotype [R^{N} or *(C)ce*^{s} haplotype] was found in the homozygous state in any of the donors. Variant *RHCE*ce* alleles or variant *RH* haplotypes [R^{N} or *(C)ce*^{s} haplotype] were found in compound heterozygosity with each other in four out of the 316 individuals (1.3%) (Table III). Variant *RHCE*ce* alleles or variant *RH* haplotypes [R^{N} or *(C) ce*^{s} haplotype] were found in heterozygosity with the *RHCE*ce*^{s} allele, or another common *RHCE* allele, in 42 out of the 316 individuals (13.3%).

A rare Rh phenotype, the Hr^{B} (RH34) negative phenotype, was predicted from *RHCE* molecular analysis [*(C)ce*^{s} *type 1 + RHCE*ce*^{s}*1006*] in one case.

The loss of the polymorphic hr^{S} (RH19) antigen was predicted from *RHCE* molecular analysis (*RHCE*ceEK* + normal *RHCE*cE* allele) in one case.

Common RhCE antigens were deduced as partial RhCE antigens in 17 cases (5%). These partial RhCE

Table III - Variant *RHCE* alleles or *RH* haplotypes identified among blood donors of African origin.

Variant ***RHCE*** **alleles or** ***RH*** **haplotypes**	Number	Common RhCE phenotype predicted from ***RHCE*** molecular analysis	Predicted absence of a polymorphic or high prevalence Rh antigen	Predicted expression of VS/V antigens
Variant *RHCE* alleles or *RH* haplotypes present in compound heterozygosity				
(C)ce^{S} type1 + ceAR	1	Partial C, c, e		VS & V
*(C)ce^{S} type1 + ces1006**	1	Partial C, c, e	hr^{B}, Hr^{B}	VS
ce^{s} 340 + ceTI	1	Partial c, e$^{\text{Ú}}$		VS & V
ceMO + ceTI	1	Partial c, e		/
Variant *RHCE* alleles or *RH* haplotypes present in the heterozygous state				
*RHCE*ceTI* + normal *RHCE*ce* allele	7			/
*RHCE*ceTI* + normal *RHCE*cE* allele	1	Partial e		/
*RHCE*ceMO + ce^{s}*	1	/		VS & V
*RHCE*ceMO* + normal *RHCE*ce* allele	7			/
(C)ce^{S} type1 + ce^{s}	1	Partial C		VS & V
(C)ce^{S} type1 + normal *RHCE*ce* allele	5	Partial C		VS & V
(C)ce^{S} type1 + normal *RHCE*Ce* allele	2	Partial c		VS & V
*RHCE*ce^{s} 1006* + normal *RHCE*Ce* allele	2	Partial c		VS
*RHCE*ce^{s} 1006* + normal *RHCE*ce* allele	2			VS
*RHCE*ceAR + ce^{s}*	1	/		VS & V
*RHCE*ceAR* + normal *RHCE*Ce* allele	1	Partial c		V
*RHCE*ceAR* + normal *RHCE*ce* allele	3			V
*RHCE*ceEK* + normal *RHCE*cE* allele	1	Partial e	hr^{s}	/
*RHCE*ceEK* + normal *RHCE*ce* allele	3			/
*RHCE*ceBI* + normal *RHCE*ce* allele	1			/
*RHCE*ce^{s}340 + ces*	1	/		VS & V
*RHCE*ce^{s}340* + normal *RHCE*ce* allele	1			VS & V
*RHCE*cE(916)* + normal *RHCE*cE* allele	1			/
RN + normal *RHCE* allele	1			/

*Complete RH genotype: association of *RHD*DIII type 5* linked to *RHCE*ce^{s}1006* with the *(C)ce^{s} type 1* haplotype; $^{\text{Q}}$RhCE antigen was considered as partial when it was encoded by two variant *RHCE* alleles present in compound heterozygosity.

antigens were partial C, partial c, and partial e in eight, nine, and six cases, respectively.

Expression of the VS antigen could be predicted from *RHCE* molecular analysis in 109 cases (34.5%). Expression of low prevalence V antigen predicted from *RHCE* molecular analysis is reported in Table III.

Variant *RH* alleles demonstrating linkage

Determination of the complete *RH* genotype showed linkage of specific variant *RHD* alleles with specific *RHCE* alleles. Linkage of *RHD*weak D type 4.2.2* with *RHCE*ceAR* was found in six blood donors; that of *RHD*DIII type 5* with *RHCE*ce^{s}1006* was found in five blood donors, and that of *RHD*DOL-2* with *RHCE*ceBI* was found in one blood donor. *RHD*DIV-a2* was found to be linked to *RHCE*ceTI* in five donors and to *RHCE*ce* in one donor.

Considering the results of *RHD* and *RHCE* gene analyses together, donors were predicted to have a rare Rh phenotype in two cases. The first donor's complete *RH* genotype was shown to be the combination of *RHD*weak D type 4.2.2* linked to *RHCE*ceAR* with the *(C)ce^{s} type 1* haplotype, predicting the production of partial D, C, c, and e antigens. The second donor's complete *RH* genotype was shown to be the combination of *RHD*DIII type 5* linked to *RHCE*ce^{s}1006* with the *(C)ce^{s} type 1* haplotype, predicting the production of partial D, C, c, and e antigens, and the loss of the hr^{B} and Hr^{B} antigens (described above).

Discussion

In this study we evaluated whether *RH* genotyping should become part of systematic testing among blood donors of African origin. The experience of our laboratory in terms of *RH* molecular testing allowed us to

determine the frequency of RhCE variants in individuals of African origin in a previous study[21]. However, that study included individuals whose samples were referred to the National Reference Centre for Blood Groups because of a depressed RhCE phenotype, antibodies against RhCE antigens in patients expressing the corresponding antigens, or antibodies against a high-prevalence Rh antigen in patients with a depressed e phenotype. That study prompted us to evaluate the frequency of individuals of African origin expressing partial Rh antigens who are, consequently, at risk of producing anti-Rh antibodies, by studying a blood donor population. The blood donors were selected based on their having the Fy(a−b−) phenotype, which was taken to indicate their African origin[28]. The frequency of the common Rh antigens in our studied population was actually in accordance with published data related to the African population[29].

Regarding *RHD* molecular analysis performed systematically, Dñ status was associated with a complete *RHD* deletion or the silent *RHD*Psi* allele in 67% and 19.4% of cases, respectively. Although a limited number of Dñ donors were investigated in our work (36 individuals), these data were unexpected since a previous study had found that Dñ status was related to the silent *RHD*Psi* allele in 66% of cases in a population of African origin[30]. Whether this difference is due only to the number of samples tested, or to other parameters such as the selection of individuals based on their Fy(a−b−) phenotype, remains to be investigated. Among the 280 D+ donors with normal D expression, *RHD* molecular analysis allowed the prediction of a partial D in 11% of cases. This calculation included the *RHD*weak D type 4.0* and the *RHD*weak D type 4.2.2.* alleles that we recently documented as producing a partial weak D because of alloanti-D in patients expressing this type of weak D (in press). The most frequent variant *RHD* alleles found in blood donors whose D phenotype was corrected as a partial D phenotype were the *RHD*DAU-3* allele, the *RHD*weak D type 4.0* allele, the *RHD*weak D type 4.2.2* allele, the *RHD*DIV-a2* allele and the *RHD*DIII type 5* allele. No variant *RHD* allele associated with the expression of a weak D, according to the rhesus base classification, was found in this study. Taken together, our data suggest that *RHD* molecular analysis, performed systematically even when D reactivity is normal, may be informative in this population because of the frequency of individuals expressing a partial D. Advice could, therefore, be provided to donors expressing a partial D in order to prevent anti-D alloimmunisation (transfusion with D− RBC units, and anti-D immunoprophylaxis for pregnant women, if necessary).

With regards to systematic molecular analysis of *RHCE,* variant *RHCE* alleles or variant *RH* haplotypes [R^N or *(C)ce*^{s} haplotype] were found to be present in 14.5% of blood donors of African origin. The most frequent ones were the *(C)ce*^{S} *type1* haplotype, the *RHCE*ceTI* allele, the *RHCE*ceMO* allele, and the *RHCE*ceAR* allele, respectively. This order was different from the one we obtained in a previous study in which the most frequent variant *RHCE* alleles or variant *RH* haplotype were the R^N haplotype, the *ceMO* allele, the *(C)ce*^{s} haplotype/*ce*^{s}*1006* allele, and the *ceAR* allele[21]. These data reinforce the notion that frequencies of variant alleles may be calculated differently in different situations, among the same population, because of a recruitment biais (blood donors, individuals with depressed antigen expression, patients, etc.). Overall, *RHCE* molecular analysis performed systematically on samples from blood donors of African origin may identify rare Rh phenotypes. Classically, a rare Rh phenotype associated with the loss of a high-prevalence Rh antigen may be suspected when weakened RhCE antigen expression is noted. However, it may be phenotypically "silent" when using usual serological methods. *RHCE* molecular analysis gave us the opportunity to identify a rare Rh phenotype associated with the loss of a high prevalence Rh antigen (Hr^B or RH34) in one out of 316 donors (0.3%). This frequency is in accordance with the definition of a rare phenotype, classically defined as a phenotype occurring at a frequency lower than 0.4%[31]. Collectively, our data suggest that *RHCE* molecular analysis performed systematically in blood donors may be efficient for predicting the expression of partial common RhCE antigens, estimated at 5% of cases, associated or not with a rare Rh phenotype.

Whether systematic *RH* molecular analysis performed among blood donors of African origin may improve transfusion therapy of patients of the same origin has yet to be determined. However, the answer is not unequivocal. First, it should be emphasised that there is genetic heterogeneity in terms of variant *RH* alleles among the African population (e.g. the R^N haplotype is preferentially found among the Peul population in West Africa). Second, our data showed that only by screening very large populations of donors may the transfusion needs of patients producing anti-Rh be met. The cost/effectiveness of such a decision requires evaluation. Finally, the donor *RHCE* molecular approach should be discussed according to the type of patients needing transfusion (non-haematological patients, polytransfused patients, etc.). Our recommendation is that medical centres caring for patients at risk of anti-Rh alloimmunisation, in particular patients with sickle cell disease, should provide red blood cell units not selected only on RhCE phenotype matching, but also on *RHCE* genotype matching.

With regards to *RH* genotyping, molecular analysis may pick up specific mutations, D variants and/or RhCE variants. Determination of specific mutations may be helpful. In this study, the 733C>G mutation alone in *RHCE* exon 5, encoding a Leu245Val substitution associated with the expression of the V (RH10) and VS (RH20) antigens, was not considered as a variant *RHCE* allele. The frequency of VS antigen, encoded by different *RH* haplotypes, was found to be 34.5% in this Fy(a−b−) donor population, as deduced from molecular analysis. This is in accordance with previously published data[19]. Anti-VS has been reported to react heterogeneously with VS+ red blood cells[32]. Furthermore, the relative immunogenicity of the VS antigen has not been evaluated extensively. The impact of systematically performed molecular determination of VS antigen in a donor population of African origin should be studied, considering the risk and the potential technical problems of identification related to anti-VS alloimmunisation. Finally, whether *RHD* and *RHCE* molecular analyses should be performed together remains to be determined. In our opinion, the notion of haplotype (*RHD* allele inherited together with *RHCE* allele) should be taken into account and lead to a systematic global *RHD* and *RHCE* molecular analysis. The frequency of variant *RH* alleles among the population of African origin favours this approach. The availability of high-throughput DNA analysis platforms may also argue in favour of such a decision. However, the choice of devices may depend on the different types of Rh variants investigated in relation to the recruitment of the laboratory involved.

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Conflict of interest disclosure

The Authors certify that they have no affiliation with or financial involvement in any organisation or entity with a direct financial interest in the subject matter or materials discussed in this manuscript (e.g., employment, consultancies, board membership, honoraria).

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Supplementary Table I - Primers and conditions of allele-specific primer amplification assays.

Primers	Sequence 5' to 3'	PCR specificity	PCR conditions
69E3CEAS	5' CTGATGACCATCCTCAGGG 3'	*RHCE*	95 C, 5 min / 10 cycles (94 C, 20s -67 C, 30s) / 21 cycles (94 C, 20s -63 C, 20s - 72 C, 20s: 30 cycles) / 72 C, 5 min / 4 C inf. 20 ng DNA, 10 µM of each primer, 200 µM of each dNTP (Amersham Biosciences, Buckinghamshire, UK), 1 µL Taq DNA Polymerase (Titanium, Clontech Laboratories, Mountain View, CA, USA) in the appropriate buffer, total reaction volume 50 µL
35I2E3DCES	5' CCTTCTCACCCCCAGTATTC 3'	C340	
68I2E3VMAYS	5' CCTTCTCACCCCCAGTATTT 3'	T340	
88TI5'NCS	5' ATAGTCCCTCTGCTTCCG 3'	*RHCE/RHD*	
89TIEI1AS	5' CCAATGAACTCTCACCTTG 3'	*RHCE/RHD*	
109D-EX3F	5' TCGGTGCTGATCTCAGTGGA 3'	*RHD*	94 C, 15min / 30 cycles (94 C, 30s -62 C, 30s -72 C, 30s) / 72 C, 10 min / 4 C inf 50 ng DNA, 12 to 24 µM specific primers, 8 µM GH1 primers, 200 µM of each dNTP (Amersham Biosciences, Buckinghamshire, UK), 0.4 µL HotStarTaq (Qiagen, Hilden, Germany) in the appropriate buffer, total reaction volume 25 µL
118-D-EX3R	5' ACTGATGACCATCCTCAGGT 3'	455A	
110CE-EX3R	5' ACTGATGACCATCCTCAGGG 3'	455C	
49I5E5CEAS	5' TCACCATGCTGATCTTCCT 3'	*RHCE*	95 C, 15 min / 28 cycles (94 C, 30s -61 C, 30s -72 C, 1 min) / 72 C, 10 min / 4 C inf 50 ng DNA, 24 µM of specific primers, 6 µM GH1 primers, 200 µM of each dNTP (Amersham Biosciences, Buckinghamshire, UK), 0.3 µL HotStarTaq (Qiagen, Hilden, Germany) in the appropriate buffer, total reaction volume 25 µL
18EX4CES	5' ACTACCACATGAACCTGAG 3'	*RHCE*	
17EX4DS	5'GACTACCACATGAACATGAT 3'	R^N	
GH1sense	5' TGCCTTCCCAACCATTCCCTTA 3'	*GH1*	
GH1antisense	5'CCACTCACGGATTTCTGTTGTGTTTC3'	*GH1*	

Supplementary Table II - Primers and conditions of exon amplification assays.

Primers	Sequence 5' to 3'	Specificity	PCR assays	PCR conditions
40NC5'DS	5' CTCCATAGAGAGGCCAGCACAA 3'	*RHD*	*RHD* exon 1	95 C 5 min: 1 cycle, (95 C 30s, 63 C 30s, 72 C 30s: 30 cycles), 72 C 5 min: 1 cycle
54I1DAS	5' TGCTATTTGCTCCTGTGACCACTT 3'	*RHD*		
135D2S	5'TGACGAGTGAAACTCTATCTCGAT 3'	*RHD*	*RHD* exon 2	95 C 10 min: 1 cycle, (92 C 20s, 64 C 30s, 68 C 1 min 30s: 40 cycles), 72 C 5 min: 1 cycle
136DC2AS	5' GGCATGTCTATTTCTCTCTGTCTAAT 3'	*RHD/RHCE*		
138D3S	5' GTCGTCCTGGCTCTCCCTCTCT 3'	*RHD*	*RHD* exon 3	95 C 10 min: 1 cycle, (92 C 20s, 64 C 30s, 68 C 1 min 30s: 40 cycles), 72 C 5 min: 1 cycle
139DC3AS	5' CTTTTCTCCCAGGTCCCTCCT 3'	*RHD/RHCE*		
148DC4S	5'GCCGACACTCACTGCTCTTAC 3'	*RHD/RHCE*	*RHD* exon 4	95 C 10 min: 1 cycle (92 C 20s, 64 C 30s, 68 C 1 min 30s: 40 cycles), 72 C 5 min: 1 cycle
149D4AS	5'TGAACCTGCTCTGTGAAGTGC 3'	*RHD*		
17EX4DS	5' GACTACCACATGAACATGAT 3'	*RHD*	*RHD* exons 4-5	95 C 5 min: 1 cycle, (95 C 30s, 60 C 1 min, 72 C 1 min 30s: 30 cycles), 72 C 5 min: 1 cycle
19IN5AS	5' AATATGTGTGCTAGTCCTGT 3'	*RHD/RHCE*		
157DC6S	5'CAGGGTTGCCTTGTTCCCA 3'	*RHD/RHCE*	*RHD* exon 6	95 C 10 min: 1 cycle, (92 C 20s, 64 C 30s, 68 C 1 min30s: 40 cycles), 72 C 5 min: 1 cycle
158D6AS	5' CTTCAGCCAAAGCAGAGGAGG 3'	*RHD*		
151D7S	5'CATCCCCCTTTGGTGGCC 3'	*RHD*	*RHD* exon 7	95 C 10 min: 1 cycle, (92 C 20s, 60 C 30s, 68 C 1 min 30s: 35 cycles), 72 C 5 min: 1 cycle
152D7AS	5' AAGGTAGGGGCTGGACAG 3'	*RHD*		
141D8S	5' GGTCAGGAGTTCGAGATCAC 3'	*RHD*	*RHD* exon 8	95 C 5 min: 1 cycle, (95 C 30s, 64 C 30s, 68 C 1 min 30s: 27 cycles), 72 C 5 min: 1 cycle
142DC8AS	5' TGGCAATGGTGGAAGAAAGG 3'	*RHD/RHCE*		
145D9S	5'TGCAGTGAGCCGAGGTCAC3'	*RHD*	*RHD* exon 9	95 C 10 min: 1 cycle, (92 C 20s, 64 C 30s, 68 C 1 min 30s: 40 cycles), 72 C 5 min: 1 cycle
146DC9AS	5'CACCCGCATGTCAGACTATTTGGC3'	*RHD/RHCE*		
154DC10S	5' CAAGAGATCAAGCCAAAATCAGT 3'	*RHD/RHCE*	*RHD* exon 10	95 C 10 min: 1 cycle, (92 C 20s, 64 C 30s, 68 C 1 min 30s: 40 cycles), 72 C 5 min: 1 cycle
155D10AS	5'AGCTTACTGGATGACCACCA 3'	*RHD*		
41NC5'CES	5' CTCCATAGACAGGCCAGCACAG 3'	*RHCE*	*RHCE* exon 1	95 C 5 min: 1 cycle, (95 C 30s, 63 C 30s, 72 C 30s: 30 cycles), 72 C 5 min: 1 cycle
55I1CEAS	5' TGCTATTTGCTCCTGTGACCACTG 3'	*RHCE*		
73I2DCES	5' TCAGTCATCCTGGCTCTCC 3'	*RHD/RHCE*	*RHD/RHCE* exon 3	95 C 5 min: 1 cycle, (95 C 30s, 60 C 1 min, 72 C 1 min 30s: 30 cycles), 72 C 5 min: 1 cycle
61I3AS	5' AGGTCCCTCCTCCAGCAC 3'	*RHD/RHCE*		
18EX4CES	5' ACTACCACATGAACCTGAG 3'	*RHCE*	*RHCE* exons 4-5	95 C 5 min: 1 cycle, (95 C 30s, 62 C 30s, 72 C 30s: 25 cycles), 72 C 5 min: 1 cycle
19IN5AS	5' AATATGTGTGCTAGTCCTGT 3'	*RHD/RHCE*		
95E5CE3'S	5' CCCAAAGGAAGATCAGCAT 3'	*RHCE*	*RHCE* exon 6	95 C 5 min: 1 cycle, (95 C 30s, 68 C 3 min, 32 cycles), 72 C 5 min: 1 cycle
96E6I6AS	5' TGTCTAGTTTCTTACCGGCA 3'	*RHD/RHCE*		
97IN63'S	5' TGTTAGAAATGCTGTTAGACC 3'	*RHD/RHCE*	*RHCE* exon 7	95 C 5 min: 1 cycle, (95 C 30s, 62 C 30s, 72 C 30s: 25 cycles), 72 C 5 min: 1 cycle
99E7CE3'AS	5' CACATGCCATTGCCGTTC 3'	*RHCE*		