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Monique Silvy, Julie Di Cristofaro, Sophie Beley, Kassim Papa, Michel Rits, et al.. Identification of RHCE and KEL alleles in large cohorts of Afro-Caribbean and Comorian donors by multiplex SNaPshot and fragment assays: a transfusion support for sickle cell disease patients. British Journal of Haematology, 2011, 154 (2), pp.260. 10.1111/j.1365-2141.2011.08691.x . hal-00645380

HAL Id: hal-00645380

<https://hal.science/hal-00645380>

Submitted on 28 Nov 2011

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Journal:	<i>British Journal of Haematology</i>
Manuscript ID:	BJH-2011-00181.R1
Manuscript Type:	Ordinary Papers
Date Submitted by the Author:	17-Mar-2011
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Key Words:	BLOOD TRANSFUSION, MOLECULAR BIOLOGY, RED CELL ANTIGENS, SICKLE CELL DISEASE

Running head: *RHCE* and *KEL* genotyping

BJH-2011-00181(revised)

Identification of *RHCE* and *KEL* alleles in large cohorts of Afro-Caribbean and Comorian donors by multiplex SNaPshot and fragment assays: a transfusion support for sickle cell disease patients.

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This study was supported by Établissement Français du Sang, Paris, France (all co-authors).

The authors declare that they have *no conflict of interest* in the subject matter of their manuscript.

ABSTRACT

To lower the alloimmunization risk following transfusion in blacks, we developed two genotyping assays for large-scale screening of Comorian and Afro-Caribbean donors. One was a multiplex SNaPshot assay designed to identify *ce^s(340)*, *ce^{MO/AR/EK/BI/SM}*, *ce^s*, *ce^s(1006)* and *KEL*6/*7* alleles. The other was a multiplex fragment assay designed to detect *RHD*, *RHD ψ* and *RHCE*C* and 455A>C transversion consistent with (*C*)*ce^s* Type 1 and *DIII Type5 ce^s*.

Variant *RHCE*ce* alleles or *RH* haplotypes were detected in 58.69% of Comorians and 41.23% of Afro-Caribbeans. The *ce^s* allele, (*C*)*ce^s* Type 1, and *DIII Type 5 ce^s* haplotypes were identified respectively in 39.13%, 14.67% and 4.88% of Comorians and 32.23%, 5.28% and 1.76% of Afro-Caribbeans. Genotypes consistent with partial D, C, c and/or e antigen expression were observed in 26.08% of Comorians and 14.69% of Afro-Caribbeans. No homozygous genotype corresponding to the RH:-18, -34, and -46 phenotypes were found. However, over 50% of genotypes produced low-prevalence antigens at risk for negative recipients, i.e., V, VS, JAL, and/or KEL6. One new variant *RHCE*ce^s(712)* allele was identified.

This is the first determination of variant *RHCE* and *KEL* allele frequencies. Results indicate most suitable targets for molecular assay screening to optimize use of compatible blood units and lower immunization risk.

Abstract = 200 words

KEY WORDS: *RHCE* and *KEL* gene variants, SNaPshot, genotype, population with African origin.

ABBREVIATIONS: SNP(s) = single nucleotide polymorphism (s); bp = base pairs; rfu(s) = relative fluorescence unit(s).

INTRODUCTION

The risk of post-transfusion alloimmunization increases with donor exposure and degree of donor/recipient red blood cell (RBC) antigen disparity (Higgins & Sloan 2008). The risk is particularly high for minority racial groups such as persons of African descent living in Europe who harbor molecular variants compared to European blood donors (Aygun, *et al*, 2002; Daniels 2002; Noizat-Pirenne 2003). Sick cell disease (SCD) patients undergoing long-term transfusion therapy have a higher risk of alloimmunization than the general population: 20 to 40 % *versus* 5% (Flickinger 2006). For SCD patients, alloimmunization has severe clinical consequences not only by increasing the difficulty of finding compatible blood but also by causing hemolytic transfusion reaction and autoantibody production and reducing the longevity of transfused RBCs (Aygun *et al*, 2002). Consistent with the fact that blacks harbor more molecular Rh variants than European blood donors (Avent & Reid 2000), it has been observed that more than 60% of alloantibodies in SCD patients are directed against Rh antigens (Chou & Westhoff 2009; Isaak, *et al*, 2006).

Two strategies that have been implemented in several countries to lower the incidence of alloantibody production in SCD patients, are extended Rh and Kell phenotype matching and intra-ethnic blood transfusions (Sosler *et al*, 1993; Wayne *et al*, 1993). Although these approaches seem to have positive effects to reduce alloimmunization (Vichinsky *et al*, 2001; Tahhan *et al*, 1994), it appears that some SCD patients still produce anti-D, -C , -c and -e despite having type D+, C+, c+ and e+ RBCs respectively, that may be incorrectly classified as auto antibodies (Westhoff 2008). Indeed, molecular analysis of *RHD*, *RHCE*, *KEL* and *MNS* genes in SCD patients has identified nucleotide polymorphisms showing that Rh, Kel and Glycophorin B polypeptides differ from conventional amino-acid sequences indicating that serum antibodies are allo-type.

Three types of variant antigens are encountered : (i) absence of high-prevalence antigens resulting from homozygous alleles, haplotypes or gene deletion, (ii) partial antigens (c, e, C, D, U^{var}), and (iii) expression of low-prevalence antigens (see Table 1). These phenotypes are generally recognized once the allo-immunization is elicited against missing epitopes or when a transfusion reaction has occurred.

These antigen variants hardly recognized by current serological reagents, can be well identified by molecular tools. Despite this advance and different genotype reports on Afro-Caribbean and African American black individuals notably with sickle cell disease, guidelines on the frequencies of variant *RHCE* alleles and *KEL**6/*7 alleles from large cohorts of

African origin would be useful to prevent alloimmunization and improve transfusion therapy in countries with large African immigrant populations.

Our transfusion center is located in the southern French department of Bouches-du-Rhône that has a large Comorian and Afro-Caribbean community (Chiaroni *et al*, 2004). The presence of this large minority population poses a major transfusional challenge because of the high frequency of blood diseases such as SCD. To allow routine evaluation of alloimmunization risk, we have developed two simple and clinically useful genotyping assays based on the well-known SNaPshot method (Di Cristofaro *et al*, 2010; Silvy *et al*, 2011). One is a multiplex primer extension assay (SNaPshot assay) allowing analysis of nucleotides at key positions 340, 667, 712, 733 and 1006 of the *RHCE* gene and position 1910 of the *KEL* gene designed to identify *ce^s*(340), *ceMO/AR/EK/BI/SM*, *ce^s*, *ce^s*(1006) and *KEL**6/*7 alleles. The other is a multiplex fragment assay allowing the detection of the presence of *RHD*, *RHD ψ* and *RHCE**C genes as well as (C)*ce^s* Type 1 and *DIII* Type 5 *ce^s* haplotypes (Pham *et al*, 2009b; Poulter *et al*, 1996; Singleton *et al*, 2000).

The purpose of this report is to describe the results of a prospective study carried out after validation of the specificity and reliability of these two molecular assays. Study was carried out on two cohorts of African descent, i.e., 184 Comorians living in the south of France and 1021 Afro-Caribbean donors from Martinique Island. Our goal was to determine their *RHCE* and *KEL* polymorphisms and to have a better knowledge of the allelic frequencies to minimize production of Rh and Kell allo antibodies, to reduce complications and improve transfusion therapy.

MATERIAL AND METHODS

Blood samples and immunohematology

Blood samples were collected on ethylenediaminetetraacetate tubes from 184 Comorian immigrants from Grande Comore Island (Njazidja) living in Marseille, France and from 1021 unrelated Afro-Caribbeans living on Martinique Island. The main selection criteria for sampling was donor origin from the Comoros or Martinique Island, i.e., place of birth mother, father and all four grandparents. The study protocol was approved by the Comité de Protection des Personnes dans la Recherche Biologique (CPPRB) in France.

Red cell serological testing was carried out at the Établissement Français du Sang (EFS) facilities in Marseille and Fort de France using commercially available MoAb reagents. Diagast reagents (Loos, France) were used to test the following specificities: anti-RhD (RH1, clones: P3x61 + P3x21223B10 + P3x290 + P3x35), anti-RhC (RH2, clones: P3x25513G8 + MS24), anti-RhE (RH3, clone: 906), anti-Rhc (RH4, clone: 951), and anti-Rhe (RH5, clones: P3GD512 + MS63). Eurobio reagents (Les Ulis, France) were used to test anti-RhD (RH1, clone: CAZ), anti-RhC (RH2, clone: MS24), anti-RhE (RH3, clone: MS35), anti-Rhc (RH4, clone: 906) and anti-Rhe (RH5, clones: MS16 + MS21 + MS63). Combinations of several anti-RhD reagents reacting with the most common partial RhD antigens in the European population particularly RhDVI were also used. Standard serological tests were performed according to the manufacturer's instructions, but indirect antiglobulin tests were not performed to detect some weak RhD variants.

Genomic DNA extraction

Genomic DNA was extracted from 200 µl of whole blood using the MagNA Pure LC DNA Isolation Kit (Roche, Meylan, France) according to the manufacturer's instructions. DNA was eluted in 100 µl water and quantified by measuring optical density.

Detection of RHCE and KEL polymorphisms by multiplex PCR, primer extension assay, and capillary electrophoresis analysis

A multiplex PCR was developed to amplify *RHCE*-gene exons 3, 5 and 7 and exon 17 of *KEL* gene (Table 2). Amplification was performed using a PTC200 thermocycler (Biorad, France) on 200 ng of genomic DNA in a final volume of 25 µl containing PCR Qiagen Master Mix, Q solution (Qiagen, Courtaboeuf, France), and primer sets (see Table 2 for final concentration). After an initial denaturation step at 95°C for 15 minutes, a total of 35 cycles were carried out

using the following sequence: denaturation at 95°C for 30 seconds, primer annealing at 57°C for 90 seconds, and polymerization at 72°C for 90 seconds. Amplification was terminated after a 10 min-extension at 72°C. Amplicons were controlled on 2% (wt/vol) agarose gel. Purification of amplified products (10 µl) were performed using 10 units of Exonuclease I and 3.3 units of shrimp alkaline phosphatase (Euromedex, Souffelweyersheim, France) for 60 minutes at 37°C followed by enzyme inactivation for 15 minutes at 80°C. The next step consisted of single-base primer extension using the SNaPshot kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol. This reaction was carried out in a final volume of 10 µl containing 3 µl of purified PCR products, 5 µl of SNaPshot ready reaction premix, and extension primers (see Table 2 for final concentration). The extension reaction included 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 30 seconds. After treatment with shrimp alkaline phosphatase, 0.5 µl of the SNaPshot primer extension reaction product was mixed with formamide and 120 LIZ ladder before analysis on a capillary sequencer (ABI PRISM3130XL, Applied Biosystems) using POP 7 polymer according to the manufacturer's instructions. Data were analyzed using GeneMapper v4.0 software (Applied Biosystems). For each SNaPshot analysis, positive and negative controls were performed by replacing genomic DNA with already genotyped DNA and H₂O (DNase/RNase free, Invitrogen) respectively.

Multiplex PCR and capillary electrophoresis analysis

A multiplex fragment assay was developed to detect the presence of *RHD*, *RHD* ψ and *RHCE***C* genes and to reveal 455A>C transversion located in *RHD* exon-3 consistent with (*C*)*ce*^s Type 1 and *DIII* Type 5 *ce*^s haplotypes. Amplification of the *CCR5* exon-2 was used as an internal control. Amplification was performed using a PTC 200 thermocycler (Biorad, France) on 200 ng of genomic DNA in a final volume of 25 µl containing PCR buffer, 3 mmol/l MgCl₂, 0.2 mmol/l of each dNTP, 1 unit of Taq DNA polymerase (Invitrogen, Cergy Pontoise, France), and primers (see Table 3 for final concentrations). Conditions for multiplex PCR were as follows: 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 59°C for 45 seconds, 72°C for 2 minutes, and 72°C for 7 minutes. As in the previous test, 1.5 µl of PCR reaction product was mixed with formamide and 500 LIZ ladder before analysis on a capillary sequencer (ABI PRISM3130XL, Applied Biosystems) using software (GeneMapper v4.0, Applied Biosystems). In each experiment, positive and negative controls were performed with already genotyped DNA and H₂O (DNase/RNase free, Invitrogen) respectively.

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Further gene analysis

To distinguish between partial *DIII* Type 5 *ce^s* and (C)*ce^s* Type 1 consistent with a 455A>C transversion located in *RHD* exon-3 revealed by multiplex fragment assay and to identify *DAR* linked to *ceAR* and *ceEK* alleles, the previously reported SNaPshot assay (Silvy et al, 2011) for detection of *weak D* and *DEL* alleles was used to analyze *RHD* positions 602, 667, 819 and 1025. To distinguish between *ceEK*, *ceBI* and *ceSM* alleles, revealed by the 712A>G transition on SNaPshot assay, and between *ceEK*, *ceBI* or *ceSM* in trans to a *ce^s* allele and *ceAR*, revealed by 712A>G and 733C>G nucleotide changes, *RHCE* exon-5 were amplified by PCR from genomic DNA using sense primer (5'-gcaacagagcaagagtcca-3', nt -310 to -292) and anti-sense primer (5'-actccccccagaaagccttg-3', nt +222 to +202). PCR products were then cloned into pGEM-T vector (Promega) and sequenced using a sequencing kit (BigDye Terminator v1.1, Applied Biosystems) and a genetic analyzer (ABI PRISM3130XL, Applied Biosystems) according to manufacturer's protocol. To distinguish between *ceBI* and *ceSM* alleles, *RHCE* exon-8 was amplified and sequenced to determine the nucleotide at position 1132. *R^N* haplotype from samples typed DCCee was investigated to identify the RH:-46, RH32 phenotype, using TaqMan assay as previously described (Tournamille *et al*, 2010).

RESULTS

Validation of assays

The first assay, i.e., multiplex PCR coupled with a single base extension reaction using genomic DNA, was designed for simultaneous determination of 6 SNPs defining 6 variant *RHCE***ce* alleles and *KEL**6/*7 alleles observed in individuals of African descent. The primer sets selected to amplify *RHCE* exons 3, 5, 7 and *KEL* exon 17 are shown in [table 2](#). For each PCR, the specificity of amplicons was evaluated in singleplex and multiplex reactions in which PCR conditions, DNA template, and primer concentrations were adjusted to obtain bands of similar intensity (data not shown). The entire assay including multiplex PCR, primer extension (see [Table 2](#)), electrophoresis, and fluorescent typing step was calibrated to obtain electrophoretic peaks of at least 500 rfus at regular intervals. Genomic DNA samples already genotyped for *RHCE***ce* variants and/or *KEL**6/*7 were used to validate the SNaPshot signal at each assay position, i.e., 340, 667, 712, 733 and 1006 of the *RHCE* and 1910 of the *KEL* gene. The validation set included *ce*^s (n=8, four homozygous samples), (*C*)*ce*^s Type 1 (n=10, one homozygous sample), *DIII* Type 5 *ce*^s (n=4, one homozygous sample), *ce*^s(340) (n=1, heterozygous form), *ceAR* (n=6, all in trans to a *ce* allele), *ceEK* (n=1, heterozygous form), and *ceMO* (n=10, one homozygous sample). Also included in the validation set were ten *KEL**7/*7, five *KEL**6/*7, and three *KEL**6/*6 samples.

The second genomic DNA assay, i.e., multiplex PCR using 8 primer sets each including one labeled primer (see [Table 3](#)), was designed for simultaneous amplification of wild type *RHD* exon-3, -4, -5 and -10, *RHD* exon-3 (455A>C), *RHD* Ψ exon-4, *RHCE***C* intron-2, and *CCR5* exon-2 as positive control. After optimization of singleplex and multiplex reaction conditions as described above, multiplex fragment signals were validated using a validation set composed of genomic DNA samples genotyped *RHD* Ψ (n = 6, one of them with *RHCE***C* allele), (*C*)*ce*^s Type 1 (n = 10, one with *RHCE***C* allele), *RHD* deletion (*del/del*, n = 6, four of them with *RHCE***C* allele), and *RHD* (n=10, five with *RHCE***C* allele).

Results obtained with both assays were fully concordant with known *RHDCE* and *KEL* genotypes. Typical GeneMapper electropherograms obtained after capillary electrophoresis in a POP7 polymer system are presented in figure 1 showing the relevance of the two molecular analyses at polymorphic positions 340C>T, 667G>T, 712A>G, 733C>G and 1006G>T of the *RHCE* gene and 1910G>T of the *KEL* genes (panel A) and wild type *RHD* exon-3, -4, -5 and -10, *RHD* exon-3 (455A>C), *RHD* Ψ exon-4, *RHCE***C* intron-2 and *CCR5* exon-2 as positive control (panel B). Electrophoregram interpretations are given in

table 4. For all samples, genotype determination was based on reading the electropherogram positions taking into account the observed RhDCE phenotype. Altogether, these results validated both multiplex assays.

Prospective study in two cohorts of African descent

Prospective study starting with the SNaPShot assay was carried out on a total of 184 Comorian and 1021 Afro-Caribbean donors RhDCE phenotyped with commercial MoAb reagents. In a second step, DNA samples presenting nucleotide changes at positions 733C>G and 1006G>T were investigated using the multiplex fragment assay to identify active and inactive *RHD* molecular background, *RHCE**C gene, and 455A>C transversion located in *RHD* exon-3 consistent with (C)*ce*^s Type 1 and *DIII* Type 5 *ce*^s haplotypes.

Phenotype findings showed that 11 (5.97%) Comorians and 92 (9.01%) Afro-Caribbeans were RhD negative. As expected, the RhDCe, DcE, Dce and dce phenotypes were the most frequent in both cohorts, resulting from *DCe*(*R*₁), *DcE*(*R*₂), *Dce*(*R*₀) and *dce*(*r*) haplotypes that are the most frequent in Africans populations (data not shown). Genotype findings (based on screening of polymorphism positions 340, 667, 712, 733 and 1006 of *RHCE* gene and on *RH* haplotype analysis) were all consistent with serologic results for RhDCE status and showed that 108 (58.69%) Comorians and 421 (41.23%) Afro-Caribbeans carried at least one variant *RHCE***ce* allele or *RH* haplotype (see Table 5).

The most frequent variants were the classic *ce*^s allele identified in 72 (39.13%) Comorians and 330 (32.32%) Afro-Caribbeans, (C)*ce*^s Type 1 (14.67% and 5.28%, respectively) and *DIII* Type 5 *ce*^s haplotypes (4.88% and 1.76%, respectively). Variant *RHCE***ce* alleles or *RH* haplotypes were found in compound heterozygosity with each other in only 16 (8.69%) Comorians and 51 (4.99%) Afro-Caribbeans.

Only 48 (26.08%) Comorians and 150 (14.69%) Afro-Caribbeans showed genotypes consistent with likely partial D, C, c and/or e antigen expression resulting from *DARceAR*, *DIII* Type 5 *ce*^s, and (C)*ce*^s Type 1 haplotypes and/or variant *RHCE***ce* alleles, in the absence of conventional *RHCE***ce* or *RHCE***Ce* in trans.

No homozygous genotype corresponding to the absence of high-prevalence antigens, i.e., RH:-18, -34 and -46 phenotype, was found. Conversely, 116 (63%) Comorians and 496 (48.60%) Afro-Caribbeans expressed low-prevalence antigens resulting from variant *RHCE* alleles and/or *KEL**6 allele. The most frequent alleles encoded VS, V and *KEL*6. *KEL**6/*7 analysis revealed that 41 (22.28%) Comorians and 158 (15.47%) Afro-Caribbeans carried at

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3 least one *KEL**6 allele. Two Comorian and 9 Afro-Caribbean samples were homozygous
4 *KEL**6/*6 with the rare Js(a+b-) phenotype.
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7 A novel variant *RHCE***ce* allele was identified in one Afro-Caribbean sample with a
8 RhCce phenotype. It was found to share the 733C>G transversion in collinear association
9 with the 712A>G transition in *RHCE* exon-5, after sequencing of 10 *RHCE* exons. These two
10 nucleotide changes are consistent with an *RHCE***ce* (238Val, 245Val) allele named
11 *RHCE***ce*^s (712). Testing to rule out high-prevalence antigens could not be performed due to
12 the presence of conventional *RHCE***Ce* in trans encoding a normal e antigen. Nevertheless,
13 the likelihood of VS low-incidence antigen is great since VS expression requires Leu245Val
14 in an *RHCE* protein encoded by the guanine at position 733 in *RHCE* exon 5.
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23 *Experimental allele frequencies*

24 Based on these genotype results, the experimental frequencies of variant *RHCE***ce*
25 alleles, *RH* haplotypes, and *KEL**6/*7 alleles were calculated for each cohort. As shown in
26 table 6, these gene features were consistently more frequent in Comorians than in Afro-
27 Caribbeans. The most frequent were the *ce*^s allele that was found in 20.38% of Comorians
28 versus 17.82% of Afro-Caribbeans, the (C)*ce*^s Type 1 haplotype in 7.33% versus 2.64%, and
29 the *DIII* Type5 *ce*^s haplotype in 2.44% versus 0.88%. All other experimental allele
30 frequencies were lower than one percent except for *ceMO* allele that was found 1.63% in
31 Comorians. The frequency of *KEL**6 allele expressing low-prevalence antigen was 11.68% in
32 Comorians versus 8.18% in Afro-Caribbeans.
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DISCUSSION

Preventing alloimmunization is a daily concern in transfusion medicine particularly for patients with diseases such as SCD that require long-term transfusion therapy. Developing molecular tools for simultaneous detection of various alloimmunization risk factors including presence/absence of high- and low-prevalence antigens as well as expression of partial antigens remains a major challenge. All recent reports have underlined the value of extensive molecular typing of recipients and donors (Hillyer *et al*, 2008; Reid 2007; Westhoff 2008). For SCD patients, previous studies indicate that molecular typing can reduce labor and reagent costs and to enhance the benefit of transfusion therapy (Guelsin *et al*, 2010; Pham *et al*, 2011; Ribeiro *et al*, 2009).

The present study describes two genotyping assays. The first is a multiplex SNaPshot assay designed for simultaneous analysis of 6 key SNPs defining a spectrum of 6 variant *RHCE*ce* alleles and *KEL*6/*7* alleles. All targeted variant *RHCE*ce* alleles are considered in Noizat-Pirenne guidelines as prevalent or clinically relevant for molecular identification of RhCE phenotypes in individuals of African descent at risk for alloimmunization against low- and high-incidence antigens (Noizat-Pirenne *et al*, 2002). *KEL6 (Js^a)*, which was first reported in 1958 (Giblett 1958), occurs in 20% of Afro-Americans versus less than 1% in the rest of the populations and has been implicated in immunogenic responses and fetal anemia. The second assay described here is a multiplex fragment assay allowing detection of *RHD*, *RHDψ* and *RHCE*C* genes as well as the 455A>C transversion consistent with (*C*)*ce^s* Type 1 and *DIII Type 5 ce^s* haplotypes. Found exclusively in blacks, (*C*)*ce^s* Type 1 encodes weak partial C, partial c, weak partial e, VS, and Rh42 (Daniels *et al*, 1998; Faas *et al*, 1997; Pham *et al*, 2009a). Since (*C*)*ce^s* Type 1 does not produce hr^B and Hr^B high-prevalence antigens, it induces in the homozygous state, the rare RH:-34 phenotype with risk of transfusion deadlock (Noizat-Pirenne *et al*, 2002; Pham *et al*, 2011). *DIII Type 5 ce^s* is relevant to transfusion because it can lead to production of alloanti-D and alloanti-hr^B and anti-Hr^B in the absence of conventional *RHCE*ce* or *Ce* in trans (Westhoff *et al*, 2010). In addition to these two genotyping assays, a TaqMan assay was used to identify *R^N* homozygous consistent with the lack of expression of the RH46 high-prevalence antigen and the appearance of the RH32 low-incidence antigen (RH:32,-46 phenotype) (Le Pennec *et al*, 1989; Rouillac *et al*, 1996).

After validating the specificity and reliability of these two multiplex assays, a prospective study was conducted on two cohorts of unrelated donors including 184 Comorians from Grande Comore Island and 1021 Afro-Caribbeans from Martinique Island.

These two cohorts of African descent present different rates of racial admixture and are representative of diversity in the general population in the two locations. As expected, our results showed a wide diversity of *RHDCE* and *KEL* genotypes in both Comorians and Afro-Caribbeans. More than 50 different *RH* genotypes were found, confirming the heterogeneous nature of *RH* genes. Variant *RHCE* alleles and *RH* haplotypes expressing low-incidence antigens were usually present in heterozygote with another common *RHCE* allele. More than one out of two genotypes produced V, VS, JAL and/or KEL6 low-incidence antigens posing a risk for negative recipients. However, no homozygous R^N and no *ceSM* alleles expressing RH32 and RH49(STEM) respectively were detected. A clear implication of these findings is that intra-ethnic blood transfusion does not rule out the need for extended matching to lower the risk of alloimmunization due to low-prevalence antigens.

Findings showed that 26.08% of Comorians and 14.69% of Afro-Caribbeans expressed partial D, C, c, and/or e antigens resulting from *DAR*, *DIII Type 5 ce^s* and *(C)ce^s* haplotypes and/or variant *RHCE*ce* alleles. Assuming that the *ce^s* allele has 48C (position not tested) and 733G, it can be estimated that more than half, i.e., 14.67% and 8.81% respectively, do not express the hr^B high-prevalence antigen. Half of these were hr^B- and E negative, a valuable phenotype for SCD patients. Finding suitable blood for patients with anti-hr^B can be problematic since anti-hr^B antibodies produced by patients with different molecular background are not always compatible with all hr^B- red cells (Reid *et al*, 1997; Vege & Westhoff 2006). Only three donors heterozygous for *ceMO* or *ceAR* in trans to a *RHCE*ce* allele correlated with the lack of the hr^S high-prevalence antigen. Anti-hr^S antibody is clinically relevant since it has been implicated in a number of transfusion fatalities (Noizat-Pirenne *et al*, 2002). Once again, anti-hr^S are not always compatible with all hr^S- red cells since negative hr^S phenotypes may be encoded by different *RHCE*ce* alleles (*ceMO/AR/EK/BI/SM*) (Noizat-Pirenne *et al*, 2002; Noizat-Pirenne *et al*, 2001). Two Comorians and 9 Afro-Caribbeans displayed a complex difficult-to-predict mosaic-type that resulted from variant *RHCE*ce* alleles and *RH* haplotypes present in compound heterozygosity with each other. To further complicate matters, the partial RhD phenotype occurred in 6 Afro-Caribbeans genotyped *dce/DIII Type 5 ce^s* (n=5) or *RHD Ψ ce/DARceAR*, (n=1) though it must be stressed that the *DIVaceTI* and *DIII Type 5 ceTI* haplotypes were not investigated (Hemker *et al*, 1999; Westhoff *et al*, 2010). These findings again underline the need for donor/recipient RH matching, which can only be achieved by DNA analysis, to prevent alloimmunization.

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Evidence that ce^s , $ce^s(340)$, $ceMO$, $ceAR/BI/EK$, $(C)ce^s$ Type 1, $DIII$ Type 5 ce^s and $ce^s(1006)$ may induce potentially immunogenic partial phenotypes and/or expression of low-prevalence antigens in Afro-Caribbeans and Comorians led us to evaluate the experimental frequency of these alleles. The incidence of rare phenotypes in the Afro-Caribbean was deduced from the frequency of the alleles at homozygous state: RH:-31 [ce^s]: 1/31; Js (a+b-) [$KEL*6$]: 1/149; RH:-34 [(C) ce^s Type 1, $DIII$ Type 5 ce^s , $ce^s(1006)$]: 1/742; RH:-18 [$ceAR/BI/EK$]: 1/13,211; RH:-19 [$ceMO$]: 1/24,414 and RH:-57 [$ce^s(340)$]: 1/4x10⁶ (see Tables 1 and 6). In the Comorian cohort, the incidence of the corresponding phenotypes was RH:-31: 1/24; Js (a+b-): 1/73; RH:-34: 1/99; RH:-18: 1/5,486; RH:-19: 1/3,763 and RH:-57: 1/137,000. Having a rate of racial admixture lower than Afro-Caribbeans, Comorians logically showed higher frequencies of rare phenotypes. No homozygous R^N genotype was found in either cohort. These frequency data clearly indicate priority targets which must be first investigated in donors and patients to optimize use of compatible units and improve transfusion. However, the cost of molecular investigations remains limiting factor.

To our knowledge, this is the first report describing molecular identification of $RHCE$ and KEL variants in large representative cohorts of donors of African descent, at high level of complexity to determine the allele frequencies. Findings showed a wide diversity of genotypes with a significant percentage of donors negative for high-prevalence and partial RhDCE antigens, and donors positive for low prevalence Rh and Kell antigens. Experimental allele frequency data clearly indicated that intra-ethnic blood transfusion alone cannot prevent alloimmunization. Similarly, extended Rh antigen phenotyping cannot fully evaluate the alloimmunization risk since it may not distinguish variant Rh antigens from conventional antigens. Indeed phenotyping reveals only altered expression of conventional antigens but then DNA analysis must be performed for reliable RhCE variant typing.

This study confirms the potential benefit of RH genotyping as a complement to serologic phenotyping. Knowledge about donor genotype is essential to assessing the potential risk of alloimmunization and could allow selection of compatible blood units especially for genotyped SCD patients with antibodies to high-prevalence antigens. An additional benefit of the molecular assays in transfusion practice would be to provide molecularly defined control RBCs for antibody identification. The two multiplex assays described here and TaqMan assay for R^N homozygosity have been implemented into routine in our regional pretransfusion laboratory. We now perform more than 300 genotypes per year. Each genotyping can be obtained in 1.5 working days including hand-on time of 6 hours and machine-on time of 8 hours. Unit cost for one DNA extraction

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3 and one multiplex assay including personnel, instrument-time, and consumable costs are
4 estimated at \$37.8. When sequencing analyses are needed, two extra days are required for the
5 final genotype. Use of assays is currently restricted to patients of African descent presenting
6 atypical profiles with anti-Rh MoAbs and SCD patients, to take into account the variant
7 alleles in the research of matched RBC units. Since some phenotypes may be encoded by
8 different alleles, a high level of complexity is required to avoid alloimmunization (Reid et al,
9 1997; Noizat-Pirenne et al, 2002; Vege & Westhoff 2006). It will soon be extended to African
10 donors for selection of rare blood units for SCD patients. Further study funded by the
11 Établissement Français du Sang is under way to identify the Rh variants that may alter the Rh
12 antigenic complex to become immunogenic with production of clinically significant
13 antibodies.
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Acknowledgments: We wish to thank the staff of haematology laboratories of EFS Alpes-
25 Méditerranée for providing blood samples used in this study. We also thank Andrew Corsini
26 for proofreading the manuscript. M. Silvy developed the assays, analysed the data and wrote
27 the paper, J. Di Cristofaro developed and performed the SNaPshot assays, S. Beley performed
28 the other assays, K. Papa, M. Rits and P. Richard contributed essential blood samples, J.
29 Chiaroni contributed to design the research and P. Bailly designed the research, analysed the
30 data and wrote the paper. The authors declare that they have no conflict of interest in the
31 subject matter of their article.
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FIGURES LEGENDS

Fig. 1. GeneMapper electropherograms obtained from multiplex SNaPshot and fragment assays. Plots showing size (nt) *versus* relative fluorescence units (rfus) for two sets of genomic DNA samples and negative controls. (A) Electropherograms of SNaPshot assays showing wild type nucleotides at positions 340, 667, 712 733 and 1006 obtained in a *RHDce/ce*, *KEL*7/*7* DNA sample (top) and C/G, G/T, T/C heterozygous at positions 733, 1006 and 1910 obtained in a *RHDce/(C)ce^s* Type 1, *KEL*6/*7* DNA sample, (B) Electropherograms of multiplex fragment assays showing *RHDCe/ce* and *RHD Ψ ce/(C)ce^s* Type 1 genotypes. Nucleotide changes are indicated at SNP site. Stars indicate use of extension primer with reverse orientation. All plots were collected using POP-7 polymer under the conditions described in M&M. Nucleotide positions tested are indicated across the top of the figure.

Table 1. Specific alleles or haplotypes of the *RHD*, *CE* and *KEL* genes for absence of high-prevalence antigens and expression of altered or low-prevalence antigens

<i>RH</i> allele or haplotype	Absence of high-prevalence antigen	Expression of altered antigen	Expression of low-prevalence antigen	Reference
<i>ce^S</i>	RH31 (hr ^B)	e	RH10(V), RH20(VS)	Daniels <i>et al.</i> 1998
<i>ceAR</i>	RH18 (Hr ^S), RH19 (hr ^S)	c, e	RH10(V)	Noizat-Pirenne <i>et al.</i> 2002
<i>ceEK</i>		e	-	Peyrard <i>et al.</i> 2009
<i>ceBI</i>	RH19 (hr ^S)	e	RH49(STEM)	Halter-Hipsky <i>et al.</i> 2009
<i>ceSM</i>		e	RH49(STEM)	Halter-Hipsky <i>et al.</i> 2009
<i>ceMO</i>	RH19 (hr ^S)	e	-	Noizat-Pirenne <i>et al.</i> 2001
<i>ce^S(340)</i>	RH57 (CEST)	c, e	RH10(V), RH20(VS) RH48(JAL)	Lomas-Francis <i>et al.</i> 2009 Ong <i>et al.</i> 2009
<i>(C)ce^S</i> <i>DIII Type 5 ce^S</i> <i>ce^S(1006)</i>	RH31 (hr ^B), RH34 (Hr ^B)	C, c, e	RH20(VS)	Daniels <i>et al.</i> 1998
		D, e		Noizat-Pirenne <i>et al.</i> 2002
		e		Pham <i>et al.</i> 2009a
				Pham <i>et al.</i> 2009b
<i>R^N</i>	RH46 (Sec)	C, e	RH32	Tournamille <i>et al.</i> 2010
				Westhoff <i>et al.</i> 2010
				Le Pennec <i>et al.</i> 1989
				Rouillac <i>et al.</i> 1996
<i>DAR</i>	-	D	-	Tournamille <i>et al.</i> 2010
<i>KEL6</i>	KEL7 (Js ^b)	-	KEL6 (Js ^a)	Hemker <i>et al.</i> 1999
<i>delGYPB</i>	MNS5 (U)	-	-	Lee <i>et al.</i> 1997
<i>GYPB^S-Int5,</i> <i>GYPB^S-230</i>	MNS3 (S)	U ^{var}	-	Huang <i>et al.</i> 1987 Storry <i>et al.</i> 2003

Table 2. Primer sequences used for multiplex PCR amplification of *RHCE* and *KEL* exons and multiplex SNaPshot detection of 6 SNPs

Gene exon	Direction	5'→3' Primer sequence	PCR product size (bp)	Primer extension size (bp)	SNP site	Final concentration (nmol/l)
<i>RHCE</i> exon-3	Forward	TCTTCTATTTCCACAGAAAGTAA	301			800
	Reverse	AGGTCCCTCCTCCAGCAC				800
<i>RHCE</i> exon-5	Forward	*T ₍₁₁₎ -CCTTCTCACCCCCAGTATT	483	30	340	440
	Forward	GGGCAACAGAGCAAGAGTCC				600
	Reverse	TCACCATGCTGATCTTCCT				1200
	Forward	*T ₍₂₈₎ -GGATGTTCTGGCCAAAGT		48	667	320
	Forward	*T ₍₃₅₎ -AATCCAAAGGAAGAATGCC		54	712	520
	Reverse	*T ₍₄₁₎ -GTCACCACACTGACTGCTA		60	733	140
<i>RHCE</i> exon-7	Forward	GGGGATTACCCACATCTCCG	120			800
	Reverse	ACCCACATGCCATTGCCGTTT				800
	Reverse	*T ₍₄₆₎ -GGTGATCTCTCCAAGCAGAC		66	1006	80
<i>KEL</i> exon -17	Forward	GTACCACCCACATCCTCACC	167			1000
	Reverse	ATGGAAAGGCAGCATAATGG				1000
	Reverse	*T ₍₅₅₎ -CATGGTTGTCACAGGCG		72	1910	136

All primers were tested for potential hairpin structures and primer-dimer problems on Integrated DNA Technologies website (<http://eu.idtdna.com/analyzer/applications/oligoanalyzer>, version: 3.0, 2008). * Each extension primer was designed to anneal immediately adjacent to the nucleotide at the SNP site on either the sense or anti-sense DNA strand. Their lengths differ by addition to the 5'-end of a polyT tail so that they can be distinguished by capillary electrophoresis. All extension primers were synthesized by Eurogentec (Seraing, Belgium) and purified by HPLC to remove incomplete primer synthesis products.

Table 3. Primer sequence used for multiplex amplification of *RHD* silent genes and *RHCEC allele**

Gene exon	Direction	5'→3'PCR-primer sequence	Final concentration (nmol/l)	PCR product size (bp)
<i>RHD</i> exon-3	Forward	TCGGTGCTGATCTCAGTGGA	1000	110
	Reverse	6-FAM -ACTGATGACCATCCTCAGGG	800	
<i>RHD</i> exon-3 (455A>C)	Forward	TCGGTGCTGATCTCAGTGGA	1000	110
	Reverse	PET -ACTGATGACCATCCTCAGGT	1300	
<i>RHD</i> exon-4	Forward	NED - ATGGCAGACAAACTGGGTGTC	2200	71
	Reverse	CTGCCAAAGCCTCTACACG	2200	
<i>RHD</i> Ψ exon-4 (37 bp duplication)	Forward	NED -AACCTGGGAGGCAAATGTT	8000	250
	Reverse	AATAAAACCCAGTAAGTTCATGTGG	8000	
<i>RHD</i> exon-5	Forward	VIC -CGCCCTCTTCTTGTGGATG	70	82
	Reverse	GAACACGGCATTCTTCCTTTC	70	
<i>RHD</i> exon-10	Forward	VIC -GGATTTTAAGCAAAAGCATCCAAGAA	7600	291
	Reverse	ACTGGATGACCACCATCATAT	7600	
<i>RHCE</i> *C intron-2 (109 bp insertion)	Forward	6-FAM -CAGGGCCACCACCATTTGAAA	3000	151
	Reverse	TGGTAGCAGGCGTCTGTAAAAAAA	3000	
<i>CCR5</i> exon-2	Forward	NED -ACCTGCTCAACCTGGCCAT	200	90
	Reverse	TTCCAAAGTCCCCTGGGC	200	

All primers were tested for potential hairpin structures and primer-dimer problems on Integrated DNA Technologies website (<http://eu.idtdna.com/analyzer/applications/oligoanalyzer>, version: 3.0, 2008).

Table 4. Model for identification of variant *RHCE* and *KEL* alleles from multiplex SNaPshot and fragment assays

Exon / intron location	exon-3	exon-5			exon-7	<i>RHDCE</i> haplotype associated	exon-17
Position and nucleotide change	340 C>T*	667 G>T	712 A>G	733 C>G*	1006 G>T		1910 C>T
Amino-acid substitution	R114W	V223F	M238V	L245V	G336C		P597L
Variant allele							
<i>ce</i> ^s (340)	C>T			C>G		<i>Dce</i>	
<i>ceMO</i>		G>T				<i>Dce</i>	
<i>ceBI</i> , <i>ceSM</i>			A>G			<i>Dce</i> , <i>dce</i>	
<i>ceEK</i>			A>G			<i>DARce</i> , <i>dce</i>	
<i>ceAR</i>			A>G	C>G		<i>DARce</i> , <i>dce</i> ,	
<i>ce</i> ^s				C>G		<i>Dce</i> , <i>dce</i>	
<i>ce</i> ^s (1006)				C>G	G>T	(C) <i>ce</i> ^s ,	
						<i>DIII</i> Type 5 <i>ce</i> ^s	
<i>KEL</i> *6							C
<i>KEL</i> *7							T
<i>RHDCE</i> allele or haplotype	<i>RHD</i> exon-3	<i>RHD</i> exon-3 455A>C	<i>RHD</i> exon-4	<i>RHD</i> Ψ exon-4 37bp dup.	<i>RHD</i> exon-10	<i>RHC</i> intron-2 109 bp ins.	<i>CCR5</i> exon-2
<i>RHD</i>	+	-	+	-	+	dp	+
<i>RHD</i> deletion	-	-	-	-	-	dp	+
<i>RHD</i> Ψ	+	-	-	+	+	dp	+
** <i>(C)ce</i> ^s Type 1	-	+	-	-	+	dp	+
** <i>DIII</i> Type 5 <i>ce</i> ^s	-	+	+	-	+	dp	+
<i>RHCE</i> *C	dp	dp	dp	dp	dp	+	+

*Not strictly specific and may be found in other genotypes encoding *CeMA*, *ce*^s(697), *ce*^s(748), *ce*^s(1025) and *ce*(48, 733, 941, 1006). **In *DIII* Type 5 *ce*^s and *(C)ce*^s Type 1 haplotypes, *ce*^s referred to *ce*^s(1006). dup. = duplication, ins. = insertion and dp = depending to *RhDCE* phenotype.

To distinguish between *ceEK*, *ceBI* and *ceSM* alleles, all revealed by the 712A>G transition on SNaPshot assay and between *ceEK*, *ceBI* or *ceSM* in trans to a *ce*^s allele and *ceAR*, all revealed by 712A>G and 733C>G nucleotide changes, *RHCE* exon 5 were amplified, cloned and sequenced as indicated in M&M. To distinguish between *ceBI* and *ceSM*, *RHCE* exon-8 were also amplified and sequenced.

Table 5. RHCE and KEL genotypes observed in Afro-Caribbean and Comorian donors

RhD phenotype	RHCE phenotype or genotype					
RhD negative 92/11	ccee: 62/1	ce/ce ^s : 5/2	<u>dce/(C)ce^s</u> : 15/-	-	dce/dceEK: 1/-	<u>ce^s/ce^s(1006)</u> : -/1
	-	-	<u>**ce/(C)ce^s</u> : 1/2	-	-	<u>dce^s/(C)ce^s</u> : 1/4
	Ccee: 5/-	-	<u>dCe/(C)ce^s</u> : -/1	-	-	-
	CEce: 1/-	-	-	-	-	-
	-	-	<u>dcE/(C)ce^s</u> : 1/-	-	-	-
RhD positive 929/173	ccee: 162/20	ce/ce ^s : 177/25	<u>Dce/(C)ce^s</u> : 15/11	<u>**ce/DIII Type5 ce^s</u> : 1/- <u>Dce/DIII Type5 ce^s</u> : 5/5 <u>dce/DIII Type5 ce^s</u> : 5/-	ce/ceMO: 5/3 Dce/dceEK: 1/1 ce/ceBl: 2/- Dce/DARceAR: 4/- <u>**ce/DARceAR</u> : 1/-	<u>ce^s/ce^s</u> : 33/3 <u>ce^s/ce^s(1006)</u> : 1/- <u>Dce^s/(C)ce^s</u> : 3/4 <u>Dce^s/(C)ce^s</u> : 1/- <u>Dce^s/DIII Type5 ce^s</u> : 3/1
	Ccee: 174/39	Ce/ce ^s : 61/20 <u>Ce/ce^s(340)</u> : 1/- *Ce/ce(712, 733): 1/- Ce/ce ^s (1006): 1/1	<u>DCE/(C)ce^s</u> : 9/2	DCE/DIII Type5 ce ^s : 2/2	Ce/ceMO: 2/1 Ce/dceEK: 3/- Ce/ceBl: 1/- <u>DCE/DARceAR</u> : -/1	-
	CCee: 28/1	-	-	-	-	-
	CEce: 51/8	-	-	-	-	-
	Ecce: 109/6	<u>cE/ce^s</u> : 40/9	<u>DcE/(C)ce^s</u> : 6/2	<u>DcE/DIII Type5 ce^s</u> : 1/1	<u>cE/ceMO</u> : 1/1 <u>DcE/DARceAR</u> : 1/-	-
	EEcc: 8/-	-	-	-	-	-
	CEEc: -/1	<u>CE/ce^s</u> : -/2	<u>DCE/(C)ce^s</u> : 1/-	-	-	-
					▲ <u>DceMO/DIII Type5 ce^s</u> : 1/- ▲ <u>DceMO/(C)ce^s</u> : 1/1 <u>ce^s/ceMO</u> : 3/- <u>ce^s/ceBl</u> : 2/1 <u>Dce^s/DARceAR</u> : 2/- <u>dceEK/Dce^s(340)</u> : -/1	
KEL genotype	KEL *7/*7: 863/143		KEL *6/*7: 149/39		KEL *6/*6: 9/2	

Number of Afro-Caribbean donors (first number) and number of Comorian donors (second number). Underlined genotypes were consistent with likely partial D, C, c, and/or e antigen expressions. For samples with *DIII Type 5 ce^s*, *(C)ce^s* Type 1 designated *(C)ce^s* in this table, *DARceAR* or *DARceEK* haplotypes, the *RHD* genotype in trans were mentioned according to the RhD phenotype, fragment assay and the weak D and DEL SNaPshot assay. Variant *ce^s* allele does not exclude *ce^s(697)*, *ce^s(748)*, *ce^s(1025)* and *ce(48, 733, 941, 1006)* alleles. In *DIII Type 5 ce^s* and *(C)ce^s* haplotypes, *ce^s* referred to *ce^s(1006)*.

*Variant *RhCE*ce(712G, 733G)* allele not previously reported. *d* = deletion of *RHD* gene.

**genotype including a *RHDΨ* allele in trans.

Table 6. Frequencies of variant *RHCE* and *KEL* alleles in Afro-Caribbean and Comorian donors

Allele or haplotype	Experimental frequency		Expression of low prevalence antigens
	Afro-Caribbean (<i>n</i> =1021)	Comorian (<i>n</i> =184)	
<i>ce</i> ^S	17.82	20.38	RH: 10, 20
(C) <i>ce</i> ^S Type 1	2.64	7.33	RH: 20
DIII Type 5 <i>ce</i> ^S	0.88	2.44	RH: 20, 48
<i>ce</i> ^S (340)	0.05	0.27	RH: 10, 20
* <i>ce</i> ^S (712)	0.05	-	nt
<i>ce</i> ^S (1006)	0.15	0.27	RH: 20
<i>ce</i> MO	0.64	1.63	
DAR <i>ce</i> AR	0.39	0.54	RH: 10, 20
<i>ce</i> EK	0.24	0.54	
<i>ce</i> BI	0.24	0.27	
<i>KEL</i> *7	91.82	88.32	
<i>KEL</i> *6	8.18	11.68	KEL6

nt = not tested, *allele not previously reported.

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