

From the investigation of *RHD-CE* hybrid genes to the recognition of *RHCE* variants and *RHD* zygosity. Expanding the analysis by QMPFSF in Brazilian donors and in patients with sickle cell disease

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Background - Hybrid genes are responsible for the formation of Rh variants and are common in patients with sickle cell disease (SCD). However, it is not usually possible to detect them by conventional molecular protocols. In the present study, hybrid genes were investigated using the Quantitative Multiplex Polymerase chain reaction of Short Fluorescent Fragments (QMPFSF), a molecular protocol that quantifies the copy number of *RHD* and *RHCE* exons. In addition, we explored additional relevant information obtained with QMPFSF, such as recognition of variant *RHCE* and *RHD* zygosity.

Materials and methods - Three groups of subjects were selected for the study: patients with SCD, self-declared African descent donors (SDA), and D-negative donors. *RHD* and *RHCE* hybrid genes were investigated by the QMPFSF method. Real-time multiplex polymerase chain reaction (PCR) assay was used to confirm the copy number of the *RHD* in two samples. Cloning was performed to investigate the allele. Relative RhD antigen density was investigated by flow cytometry, and RhCE phenotyping was performed with both tube and gel methods.

Results - In the 507 samples analysed, hybrid allele frequencies were found in 20.08% of patients with SCD, in 18.22% of individuals in the SDA group, and 3.67% of D-negative donors. The SCD and SDA groups had a higher frequency of hybrid alleles, most commonly involving exon 8, with which we found an association with *c.733C>G*, a common polymorphism observed in individuals of African descent. Of note, two patients with SCD were shown to carry three gene copies, as confirmed by quantitative PCR; no increase in D expression was observed in these patients. In addition, the QMPFSF guided the investigation of 144 *RHCE* variants and *RHD* zygosity, and two novel alleles were identified.

Discussion - The QMPFSF was shown to identify hybrid alleles involved in altered Rh phenotypes in Brazilian donors and patients with SCD. The association of the hybrid *RHCE-D(8)-CE* allele with *c.733C>G* suggests this hybrid allele may be used as a marker to detect the most frequent variants found in patients with SCD.

Keywords: *hybrid gene, Rh system, sickle cell disease, Rh genotyping.*

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INTRODUCTION

The Rh system is the most complex and immunogenic blood group system, and is linked to the expression of two polymorphic genes, *RHD* and *RHCE*¹. Several molecular mechanisms, including insertions, deletions and single nucleotide variants (SNVs), have been reported to result in a diversity of Rh variant phenotypes. In addition, because *RH* genes are in close proximity, are homologous and located in opposite orientation, genetic rearrangements (i.e., exchange of genetic materials between *RHD* and *RHCE* leading to the formation of hybrid genes) may occur and many hybrid alleles have been characterised^{2,3}.

Genetic transfer from *RHD* to *RHCE* by conventional serological and molecular methods is not frequently reported, and is rather to be observed in individuals expressing the rare D-- phenotype, which is characterised by silencing of RhCE antigen expression. Conversely, transfer from *RHCE* to *RHD* is more commonly detected, notably in hemizygous samples, and results in either a D-negative or a D variant phenotype. Although D-negative is most often associated with the deletion of the entire *RHD* gene in Caucasians and with a non-functional *RHD* allele, referred to as the *RHD* pseudogene (*RHD**Ψ), in individuals of African descent, about 15% of D-negative in this latter group arise from a hybrid gene as a result of the insertion of *RHCE* segments (exons 4 to 7) in *RHD* (*RHD**01N.06 and *RHD**03N.01)⁴.

So far, more than 15 hybrid genes conferring a partial D phenotype have been described, e.g., *DIIIC*, *DVI* and *DV type2*. Due to epitope changes, they usually show discrepancy in antisera reactivity. Thus, many patients with the partial D phenotype may type as D-positive and the variant is discovered only after anti-D alloimmunisation⁵. Despite the availability of commercial antisera panels, classification of partial D by serologic methods is problematic, and molecular testing is a valuable complementary approach for characterization. However, the identification of *RHD*-*CE* hybrid genes is not a simple task, especially in compound heterozygous samples.

Many molecular approaches are used in variant *RH* allele genotyping, such as conventional PCRs, Sanger sequencing and genotyping platforms that use specific primers for *RHD* or *RHCE* genes. However, some hybrid genes cannot be detected by these standard molecular protocols. One option to detect them is cDNA analysis, but

the protocols require fresh samples for mRNA extraction and these are not usually available.

The characterization of altered *RH* alleles is of critical importance for individuals of African origin, including patients with sickle cell disease (SCD) because, as previously shown, more than 80% of patients with SCD have at least one altered *RH* allele⁶. Among the hybrid alleles in individuals with SCD, *RHD***DIIIC*-*CE*(4-7)-D and *RHD**D-*CE*(4-7)-D, which are linked to *RHCE***ceVS.03* allele compounding the (C)*ce*^s type 1 and (C)*ce*^s type 2 haplotypes, respectively, are quite common⁷. This haplotype results in a complex phenotype, with partial C and e antigens and absence of highly prevalent hr^B and Hr^B antigens⁴. As it is not recognised by serological tests, studies have shown the important impact of the (C)*ce*^s haplotype in the transfusion of patients with SCD and the requirement for a molecular approach^{6,8-10}. Recognition of *RHD* and *RHCE* variants in patients with SCD has become increasingly important, as several studies have shown that even by providing prophylactic CEK-matched transfusions, these patients continue to develop Rh antibodies as a consequence of Rh variant phenotypes^{11,12}.

The present study investigated hybrid alleles in Brazilian donors and patients with SCD using the Quantitative Multiplex Polymerase chain reaction of Short Fluorescent Fragments (QMPSF) approach, a molecular protocol that quantifies the copy number of *RHD* and *RHCE* exons¹³. In addition, we explored additional relevant information obtained with QMPSF, such as recognition of variant *RHCE* and *RHD* copies.

MATERIALS AND METHODS

Blood samples

Three different groups were enrolled in this study. Two-hundred and thirty-nine DNA samples were selected from Brazilian patients with SCD, 118 from self-declared African descent donors (SDA), and 150 from randomly selected D-negative donors. Samples were selected at Colsan (Associação Beneficente de Coleta de Sangue, São Paulo, SP, Brazil) and at the University of Campinas (UNICAMP, Campinas, SP, Brazil). All donors and patients signed an institutional informed consent form.

All DNA samples from both the SCD and the SDA groups had been previously genotyped for *RHCE* variant alleles. The data were compared with the results obtained by QMPSF.

DNA extraction

Genomic DNA was extracted from buffy coat of peripheral blood using the DNA blood mini kit (PureLink Genomic DNA Mini Kit, Invitrogen, CA, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed by the NanoDrop 2000 Spectrophotometer (Thermo Cycler, Uniscience Inc., São Paulo, SP, Brazil) and DNA samples were stored at -20°C .

Identification of *RHD-CE* hybrid genes

The samples were investigated for the presence of the *RHD-CE* hybrid genes by QMPFS as previously described¹³. Briefly, two separate reactions were performed to quantify the copy number of all exons of both *RH* genes, which contain ten primer pairs specific for the *RHD* or *RHCE* exons. Primer pairs for *HFE* and *F9* genes are used as positive amplification markers. The reaction product was mixed with deionised formamide and size standard (GeneScan™500 LIZ® Size Standard, Thermo Fisher Scientific, Horsham, UK). Polymerase chain reaction (PCR) products were separated by capillary electrophoresis (3500xL, Applied Biosystems). Each amplification generates a peak, the area of which is calculated by computer software (GeneMapper Software version 4.1, Applied Biosystems Waltham, MA, USA), and all areas were exported to a computer spreadsheet (Excel) for subsequent calculation.

Real-time quantitative polymerase chain reaction

Quantitative real-time multiplex PCR assay was used to confirm the *RHD* exon copy numbers obtained by QMPFS. Based on the protocol described by Chiu *et al.*¹⁴, a specific *RHD* region in exon 10 and the albumin gene were amplified using TaqMan System (QuantiNova Probe PCR kit, Qiagen, Hilden, Germany). Real-time quantitative PCR was carried out in an Applied Biosystems 7500 Real Time PCR Systems (Thermo Fisher Scientific, Waltham, MA, USA). The results were expressed in threshold cycle (Ct), which is the number of amplification cycles required to reach a fixed threshold of signal intensity. The Ct obtained is inversely proportional to the amount of DNA template. *RHD* gene dosage was calculated by the difference between the *RHD* Ct and albumin gene, called ΔCt . Results were analysed using a panel of samples with a known number of *RHD* alleles.

Haplotyping

Cloning of *RHCE* introns 4 to 6 was performed in a sample

genotyped as *RHCE*^{*cE}/*RHCE*^{*ce} with the c.916A>G SNV to discriminate the alleles and show in which allele this variation segregates. A long PCR product (~2.5 kb) ranging from intron 4 to intron 6, and encompassing the whole exon 5, intron 5, and exon 6 sequences, was amplified with a commercial kit (HotStarTaq Master Mix Kit, Qiagen) in the recommended conditions with *RHCE*-specific primers (forward: 5'-AAACAAATCAGCTGGGCATGGTGG-3'; reverse: 5'-TGAAGCCAATAAGAGAATGCACCA-3')¹⁵ using the sample gDNA as a template. The PCR fragment was subcloned into a commercial vector by a TOPO TA-cloning approach (TOPO TA Cloning Kit for Sequencing with pCR4-TOPO Vector, Invitrogen). The resulting product was transformed into competent cells (TOP10 Chemically Competent *E. coli*, Invitrogen) and several colonies were cultured under selective conditions. Finally, the alleles were identified by Sanger sequencing analysis using both the forward and reverse primers for sequencing specifically *RHCE* exons 5 and 6, as well as the respective flanking intronic regions.

Flow cytometry measurement of D antigen density

The relative RhD antigen density was defined by flow cytometry, by adapting the previously described protocol¹⁶ to the use of a single monoclonal IgG antibody. For standard red blood cell (RBC) samples, we selected two samples with D+C+c+E+e+ (R_1R_2) phenotype that were *RHD* homozygous, and that reacted 4+ in D typing that were tested at the beginning and at the end of tests, according to the manufacturer's instructions¹⁶. The antiserum used was the IgG D415 cell line from Immucor® with FITC-conjugated anti-human IgG as the secondary antibody (BD Pharmingen). The tests were performed in triplicate and the values calculated in the v spreadsheet (version 1.1) available on the Rhesus website¹⁷.

Phenotyping

To analyse the effect of some molecular changes on the expression of RhCE antigens, we performed phenotyping of RhCE antigens in a tube and gel test using commercial anti-C, anti-c, anti-E, anti-e reagents according to the manufacturer's instructions (Lorne Laboratories, Berkshire, UK).

Serological D typing was determined by manual gel and tube test. The gel test was performed using DG gel ABO/Rh (2D) with IgM anti-D P3x61 clone and with anti-D P3x290, P3x35, P3x61 and P3x21223B10 blend clones

(Grifols, Barcelona, Spain). D typing was performed using a tube method with anti-D MS26 +MS201 blend clones (Fresenius, Itapeperica, Brazil).

RESULTS

RHD zygosity

Using QMPF, we observed that, in our cohort, 131 and 202 samples carried one copy and two copies of the *RHD* gene, respectively. Interestingly, by this approach, two patients with SCD carrying three copies of the *RHD* gene were identified; this was further confirmed by quantitative real-time PCR. One of these samples was analysed by flow cytometry; no increase in D antigen expression was observed, presenting fluorescence intensity similar to the R_1R_2 control samples (99%).

Hybrid alleles

A total of 507 samples were analysed by QMPF. Hybrid allele frequencies were 20.08% in patients with SCD, 18.22% in individuals from the SDA group, and in 3.67% of D-negative donors. **Table I** describes the hybrid allele frequencies found among the groups.

The SCD and SDA groups had a higher frequency of hybrid alleles, and the most common hybrid alleles that alter antigen expression were the *RHD*01N.06* (7/507) and *RHD*03N.01* (7/507). In addition, hybrid alleles related to partial D antigen have been identified in patients with SCD, including *RHD*DIIC*, *RHD*DV.2* and *RHD*DIIIa-CE(9)-D*, with anti-D present in serum only with *RHD*DIIIa-CE(9)-D* which reacted 4+ with

commercial anti-D reagents by serological methods. *RHCE-D(8)-CE* and *RHD-CE(8)-D* were the most common hybrid alleles found in the SCD and SDA groups. As the coding sequences of *RHD* and *RHCE* exons 8 are identical, this rearrangement has no impact on the expression in RhD and RhCE antigens.

By investigating the *RHCE* alleles, we observed that 100% of the *RHD*01N.06* samples are linked to *RHCE*ceVS.03*. The hybrid allele *RHD*01N.04* confirmed by sequencing was found exclusively in the SDA group, while the *RHCE*ce.16* allele (*RHCE*ce(48C)-D(9)-ce*), which is commonly *cis*-associated with *RHD*Ψ*, was the most frequent hybrid allele observed in the D-negative group (*Online Supplementary Content, Table SI*).

As the primers used for QMPF target gene-specific regions/nucleotides that can be changed in some *RHCE* variants, potential allele drop-out and/or unexpected PCR amplification may provide additional information that could be useful for RH variant investigation. Taking advantage of this, samples with the *RHCE*ce.01* were detected by an additional copy of *RHCE* exon 1, samples with *RHCE*ceAR* did not amplify exons 5 and 6, and samples with *RHCE*ceEK* did not amplify exon 5. All these alleles were confirmed by sequencing.

Association of the *RHCE*ce-D(8)-ce* hybrid allele with *RHCE*ce733G*

A high number of *RHCE*ce-D(8)-ce* hybrid alleles was observed in both the SCD and the SDA groups. Although no clinical impact was observed, we found this hybrid

Table I - Frequency of hybrid alleles in patients with sickle cell disease (SCD), self-declared African (SDA) donors, and donors with D-negative phenotype

RH alleles	Hybrid alleles	SCD	SDA	D-negative donors
Not listed	<i>RHD*DIIIa-CE(9)-D</i>	1 (0.21%)	0	0
<i>RHD*DIIC</i>	<i>RHD*D-CE(3)-D</i>	1 (0.21%)	0	0
<i>RHD*DV.2</i>	<i>RHD*D-CE(5)-D</i>	1 (0.21%)	0	0
<i>RHD*01N.04</i>	<i>RHD*D-CE(3-7, 9)-D</i>	0	1 (0.42%)	0
<i>RHD*01N.06</i>	<i>RHD*D-CE(4-7)-D</i>	4 (0.84%)	3 (1.27%)	0
<i>RHD*03N.01</i>	<i>RHD*DIIIa-CEVS(4-7)-D</i>	5 (1.05%)	0	2 (0.67%)
	<i>RHCE*ce-D(9)-ce</i>	2 (0.42%)	0	0
<i>RHCE*ce.16</i>	<i>RHCE*ce48C-D(9)-ce</i>	4 (0.84%)	5 (2.12%)	9 (3.0%)
Not listed	<i>RHCE*CE-D(8)-CE</i>	59 (12.34%)	32 (13.56%)	0
Not listed	<i>RHD*D-CE(8)-D</i>	19 (3.97%)	2 (0.85%)	0
Total		96 (20.08%)	43 (18.22%)	11 (3.67%)

allele to be associated with $RHCE^{*ce733G}$, which is commonly observed in individuals of African- descent with $RHCE$ variant allele groups (Table II). Interestingly, out of the 80 samples carrying $c.733C>G$, 71 (89%) were associated with $RHCE^{*ce-D(8)-ce}$. $RHCE^{*ceVS.01}$ was the most common variant allele and showed 94.9% association with $RHCE^{*ce-D(8)-ce}$, followed by $RHCE^{*ceVS.02}$ and $RHCE^{*ceVS.03}$ showing an association of 93.8% and 75.0%, respectively, with the same hybrid allele. An analysis of all samples carrying the $RHCE^{*ce-D(8)-ce}$ allele found that 98% carried the SNV $c.733C>G$.

Table II - $RHCE$ variant alleles associated with $RHCE^{*ce-D(8)-ce}$ in patients with sickle cell disease (SCD) and self-declared African (SDA) descent donors

$RHCE$ variant	N	Associated with $RHCE^{*ce-D(8)-ce}$	% of association
$RHCE^{*ceVS.01}$	39	37	95%
$RHCE^{*ceAR}$	9	8	89%
$RHCE^{*ceVS.02}$	16	15	94%
$RHCE^{*ceVS.04}$	2	2	100%
$RHCE^{*ceVS.03}$	12	9	75%
$RHCE^{*ceEK}$	2	0	0%
Total	80	71	89%

Novel alleles

Two novel alleles were detected among the 507 samples. In a patient with SCD with an apparently normal expression of RhCE antigens, only one copy of $RHCE$ exon 6 was detected by QMPSE. On sequencing of all ten $RHCE$ exons, only the missense $c.916A>G$ SNV, which is located in exon 6, was found at the heterozygous state. This molecular change resulting in the p.Ile306Val substitution in the RhCE protein has already been described in *cis* with additional SNVs (Genbank accession number ONo65537). But this is the first report showing this SNV only, thus defining a novel $RHCE$ allele. In order to characterise this allele, a large PCR product ranging from intron 4 to intron 6 (thus spanning exons 5 and 6), the former carrying the nucleotide determining E/e allele specificity at position $c.676$, was amplified and subcloned into a plasmid vector (see above). After colony sequencing (Figure 1), it was clearly shown that the $c.916A>G$ SNV segregates with $c.676C$ (the latter being the marker of an $RHCE^{*E}$ allele), thus defining the novel $RHCE^{*cE(c.916G)}$ [or $RHCE^{*03(c.916G)}$] allele, while the other allele was $RHCE^{*ce}$ (or $RHCE^{*01}$).

The other new allele was found in two patients with SCD phenotyped as C-c+E+e- (R_2R_2) with a normal expression of the c and E antigens, but carrying RHD exon 9 into $RHCE$, characterizing the $RHCE^{*cE-D(9)-cE}$ hybrid allele. A hybrid allele related to exon 9 has been reported in the literature with the $RHCE^{*ce}$ allele ($RHCE^{*ce.16}$), which is commonly associated to $RHD^{*Y^{18}}$, while $RHCE^{*cE-D(9)-cE}$ was found to be in *cis* with a conventional RHD (Genbank accession number ONo65538).

DISCUSSION

RH variant alleles are common in patients with SCD and their investigation is important to prevent alloimmunisation. However, molecular detection of hybrid alleles is sometimes hampered by limitations of the technique. The QMPSE-based method has been shown to be reliable for characterizing D- samples, but also for identifying hybrid alleles in samples with hemizygous and compound heterozygous genes, as well as for investigating RH variants and determining RHD zygosity¹³. In this work, we investigated the presence of hybrid alleles in 507 Brazilian individuals, including patients with SCD and blood donors. In this analysis, the hybrid allele frequencies were 20.08% in patients with SCD, 18.22% in individuals from the SDA group, and 3.67% of D-negative donors. In addition, we identified one new hybrid allele. The most common hybrid allele found in our samples is not clinically relevant, as it involves exon 8 that is homologous in both RH genes. In addition, the QMPSE guided the investigation of 144 $RHCE$ variants with other molecular backgrounds and assisted in determining RHD zygosity and $RHCE^{*C/c}$ genotype.

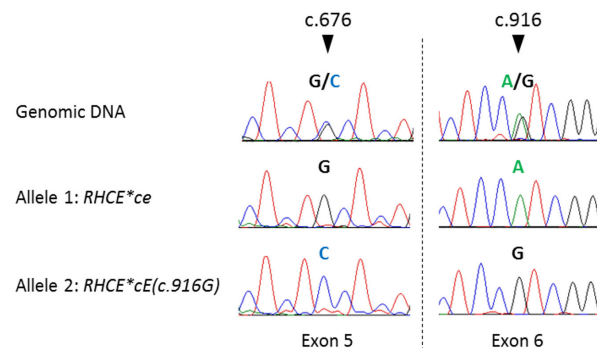


Figure 1 - Genomic Sanger sequencing analysis of $RHCE$ exons 5 and 6 and allele sequencing after cloning approach

The partial D phenotype in individuals of African ancestry is relatively common and its investigation in patients with SCD is especially important to avoid anti-D formation¹⁹. In our cohort of 239 patients with SCD, we found three samples with partial D raised from hybrid alleles: *RHD*DIIIc*, *RHD*DV.2*, and *RHD*DIIIa-CE(9)-D*. Although *DIIIc* had been previously reported in Europeans, its presence in this patient reflects the miscegenation of the Brazilian population. In a study carried out in the same region of the country, Silva *et al.*²⁰ identified a patient with SCD carrying the *RHD*Ψ/RHD*DIIIc* genotype, confirming the common observation that Brazilian patients with SCD are of mixed African and Caucasian ancestry. The hybrid *RHD*DIIIa-CE(9)-D* found in our samples was recently identified by next-generation sequencing (NGS), but without phenotypic description²¹. Insertion of *RHCE* exon 9 into *RHD* leads to the p.Glu398Val single amino acid change. Our results showed that the sample with this hybrid allele had normal D antigen expression by gel and tube tests, and anti-D was detected in the serum. The *DIIIa* and *RHD-CE(9)-D* variants have already been described separately¹⁷. It is well known that *DIIIa* is frequent in individuals of African descent, displays normal D expression, and is prone to form anti-D. Even though *RHD-CE(9)-D* variant reacts 4+ with conventional serological methods, Jakobsen *et al.*²² found one sample that showed a reduction in D antigen level quantification by flow cytometry²². Hybrid alleles related to D-negative phenotype were observed in 15 individuals, *RHD*01N.06* (*RHD*D-CE(4-7)-D* being one of the most frequent. All samples with this hybrid allele had *RHCE*ceVS.02*, constituting the (C)*ce*^s type 2 haplotype that produces partial C and e antigens, expresses VS antigen, and does not produce D, V, hr^B and Hr^B antigens, the latter being a high frequency antigen^{4,10}. In our study, (C)*ce*^s type 2 haplotype was found in four patients with SCD. Anti-C antibody was detected in one of them, who was alloimmunised also by anti-E, -Fy^a and -Jk^a. In our cohort, this hybrid gene was more frequent in patients with SCD than in Afro-Brazilian blood donors. According to our previous study²³, D-C+ screening provides a high probability of finding homozygous donors for the (C)*ce*^s haplotype, and QMPFS can be a valuable screening strategy in these selected donors.

In recent years, investigation of *RHCE* variants in patients with SCD has been the object of great interest, because:

1) such variants are frequent in this group; 2) they may be clinically relevant and result in alloimmunisation; and 3) selection of compatible donors may be complicated. An advantage of choosing the QMPFS approach as a genotyping strategy is that it guides the investigation of some *RHCE*ce-D(8)-ce* variants that are very common in patients with SCD. Although the purpose of QMPFS is to detect *RH* variants arising from hybrid alleles, the primers are located in regions where the nucleotide changes that are characteristic of some variant alleles are present, such as c.787A, c.800T in exon 5, and c.916A in exon 6. In these cases, when there is a nucleotide change, the related exon is not amplified. In addition, exon 1 amplification is specific for c.48C. Analyses of these variant profiles lead to the identification of *RHCE* variants and the *RHCE*c/RHCE*C* genotype, as observed in this study. Interestingly, a high number of samples with the *RHCE*ce-D(8)-ce* hybrid allele associated with SNV c.733C>G was observed in patients with SCD and African donors. Ninety-eight percent of the samples harbouring *RHCE*ce-D(8)-ce* also carried c.733C>G, both genetic events being common in this population. As shown in previous studies, most of the patients with SCD and African donors with *RHCE* variants also carry c.733C>G^{7,24}. Thus, by gathering the *RHCE*ce-D(8)-ce* analysis and the specificity of the primers, we were able to identify 86% of the *RHCE* variant alleles from our patients with SCD. This shows that QMPFS may be an additional tool to identify exon 8 rearrangement as a marker of variant *RHCE* alleles and their clinical relevance. On the other hand, 13/94 variant alleles, including *ceMO*, *ceAG*, *ceTI* and *ceBI* variants, were not detected.

Furthermore, as QMPFS provides the copy number of all exons, *RHD* zygosity status can be defined. Two patients with SCD carrying three copies of the *RHD* gene were identified. A previous study had also reported three *RHD* copies in a Brazilian patient with SCD after signal gain analysis of all wild-type *RHD* probe combinations from the MLPA protocol²⁵. In the Chinese population, Ji *et al.*²⁶ also described a similar case using the MLPA technique. However, they observed three copies in exons 3-10 only, suggesting a different molecular mechanism. Using flow cytometry, we observed that the presence of the additional copy does not result in increased D antigen density.

CONCLUSIONS

Hybrid alleles involved in altered Rh phenotypes (i.e., those with altered antigen expression and/or partial antigens) in African Brazilian donors and patients with SCD were easily identified using the QMPSPF-based method. Hybrid gene identification in patients with SCD is of great value in predicting partial D phenotype and RhCE variants, avoiding anti-D formation, and detecting hybrid genes related to the lack of high-frequency antigens. The hybrid gene involved in the (C)ce^s haplotype was the most common clinically-relevant hybrid allele in patients with SCD, because it encodes partial C and e antigens and does not express the high frequency antigen Hr^b that has a clinical impact on transfusion practice. In addition, we describe an interesting association of RHD exon 8 insertion in RHCE with c.733C>G, allowing the detection of the most frequent variants found in patients with SCD.

AUTHORSHIP CONTRIBUTIONS

TAPV performed serological and molecular tests, analysed the results and wrote the paper. CPA contributed to the study design and analysed the data. FL contributed to writing the paper. AJC reviewed the paper. YF standardized the methodology, analysed the results, and reviewed the paper. CB performed the molecular tests. LC designed the study, evaluated the data, and reviewed the paper.

The Authors declare no conflicts of interest.

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