Transfusion and Apheresis Science xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Transfusion and Apheresis Science

journal homepage: www.elsevier.com/locate/transci

Effectiveness of strategies to screen for blood donors with *RH* variants in a mixed population

Fabio Augusto Abreu de Almeida[a,b], Marcia Regina Dezan[a], Valeria Brito Oliveira[a,b], Cecilia Salete Alencar[b,f], Fabio Luz[a], José Eduardo Krieger[c], Alexandre Costa Pereira[c], Ester Cerdeira Sabino[b], Vanderson Rocha[d,e], Alfredo Mendrone-Júnior[b], Carla Luana Dinardo[a,b,*]

[a] *Fundação Pró-Sangue Hemocentro de São Paulo, São Paulo, Brazil*
[b] *Instituto de Medicina Tropical, Faculdade de Medicina da USP, São Paulo, Brazil*
[c] *Laboratory of Genetics and Molecular Cardiology, Heart Institute (InCor), São Paulo, Brazil*
[d] *Serviço de Hematologia, Hemoterapia e Terapia Celular Hospital das Clínicas Faculdade de Medicina da Universidade de São Paulo, Instituto do Câncer do Estado de São Paulo, Sao Paulo, Brazil*
[e] *Department of Hematology, Churchill Hospital, NHS BT, Oxford University, Oxford, United Kingdom*
[f] *Central Laboratory, Hospital das Clínicas da Faculdade de Medicina da USP, São Paulo, Brazil*

ARTICLE INFO

Keywords:
RH
Donors
RH variants
Alloimmunization

ABSTRACT

Introduction: Patients with RH variants presenting antibodies directed to RH high frequency antigens or multiple RH antibodies might, in some occasions, be better served with *RH* genotype-matched units, requiring screening for *RH* variants among blood donors. To date, strategies to identify donors with *RH* variants were restricted to selecting individuals of African descent based on self-reported race, what can be inaccurate in racially mixed population. Our goal was to: 1) Screen for donors with *RH* variants in a mixed population using self-declared race and Rh phenotype as selection criteria; and 2) Verify if including the Duffy null genotype in the screening algorithm increases its effectiveness.

Methods: Brazilian donors were included if self-declared as black and phenotyped as R0r or R1r. All individuals were genotyped for *RHCE* exons 1, 5, 6 and 7 and for the *FY*B* c. −67 T > C polymorphism in order to determine the Duffy null genotype. *RHD* variants were searched for in cases of altered *RHCE*.

Results: Among 2500 blood donors, 217 fulfilled the inclusion criteria and were enrolled. Fifty-three (24.4 %) had a predicted clinically relevant Rh phenotype (partial antigens or lack of high frequency antigens). Twelve donors (5.5 %) had a predicted RhCE phenotype lacking either hrB or hrS. Most cases with predicted lack of high frequency antigens (66.7 %) occurred in donors with the Duffy null genotype.

Conclusion: Selecting donors based on self-declared race, Rh phenotype and Duffy null genotype is feasible and effective in identifying *RH* variants lacking Rh high frequency antigens among racially mixed donors.

1. Introduction

The RH system is complex and the diversity of variant RH alleles is high, especially among people of African ancestry [1–4]. The RH system comprises 55 antigens of which D (RH1), C (RH2), E (RH3), c (RH4) and e (RH5) are the most immunogenic and relevant to transfusion practice. The *RH* locus is composed of two homologous genes: *RHD*, encoding the D antigen and *RHCE*, encoding the antigens C, E, c, e and the other antigens of the system. The frequency of structural variations (gene conversions, insertions and deletions) and single nucleotide polymorphisms involving *RHD* and *RHCE* are high, giving rise to a wide diversity of variant antigens. Rh antibodies are considered clinically relevant due to the association with hemolytic transfusion reactions and hemolytic disease of the fetus and newborn.

To date, more than 500 variant *RHD* and 80 *RHCE* have been described, encoding partial, weak or low frequency antigens and/or proteins lacking high frequency antigens. These are clinically relevant since their presence may justify the occurrence of post-transfusion alloimmunization and, potentially, post-transfusion hemolysis.

The frequency of red blood cell (RBC) alloimmunization among sickle cell disease (SCD) patients is high [5,6]. The factors underlying the predisposition to alloantibody development in these patients are multiple, but are mainly due to the red blood cell (RBC) phenotype and racial mismatch between patients and blood donors; as well as the

* Corresponding author at: Av. Dr. Enéas de Carvalho Aguiar 155, 1st floor, Pinheiros, São Paulo, SP, 05403-000, Brazil.
E-mail address: carlaluana@usp.br (C.L. Dinardo).

https://doi.org/10.1016/j.transci.2020.102720
Received 16 October 2019; Received in revised form 26 November 2019; Accepted 23 December 2019
1473-0502/

intense chronic inflammation status and high prevalence of *RH* variants among SCD patients [7–9]. It has been previously demonstrated that over 80 % of SCD patients have at least one altered RHD or RHCE allele [10], justifying the development of Rh antibodies irrespective of the use of transfusion antigen (C, c, E, e; K; Fya, Fyb; Jka, Jkb; S, s) compatible RBC units. In the presence of alloantibodies formed as a result of *RH* variants, especially if directed to high frequency antigens, SCD patients may need the transfusion of *RH*-matched units. In addition, the high prevalence of altered RH alleles begs the question of whether alloimunization preventive strategies should be first-line standard of care for these individuals.

Recent evidence has shown that the RH allele diversity and frequency in a large US cohort of SCD patients were similar to those found in black blood donors and significantly different from those found in white donors [11]. Providing SCD patients with *RH*-compatible RBC units was proved feasible with a donor pool comprised primarily of self-declared black donors [11]. This strategy, if applied prophylactically, would mitigate RBC alloimmunization in the SCD patient population as long as extended antigen-matched units were provided. Nonetheless, the costs associated with *RH* genotyping of blood donors is a major limitation of this strategy. Literature lacks more reports of screening for *RH* variants among blood donors of different populations, especially racially mixed.

The main goal of this study was to provide empirical data reporting the results of a predefined strategy to screen for blood donors with *RH* variant genotype in a mixed population using self-declared race and Rh phenotype as selection criteria. As a secondary goal, we evaluated if including the Duffy null genotype (*FY*02 N.01/FY*02 N.01*) as inclusion criteria would increase the effectiveness of the screening protocol in identifying donors with clinically relevant *RH* genotype. The *FY*02 N.01* allele is an ancestry-informative marker extremely prevalent among people of African descent that could possibly refine self-reported race as inclusion criteria for the screening protocol [12].

2. Methods

2.1. Donor recruitment

This study was conducted according to the Helsinki principles and approved by our local ethics committee. Informed consent was obtained for all participants. Two thousand five hundred blood donors registered at the reference hemocenter (Fundação Pró-Sangue São Paulo Hemocenter, São Paulo, Brazil) were selected for the study. Of these, 217 were included in the study according to the inclusion criteria, which were: 1) Black as self-declared race; 2) Blood type O; 3) RH phenotype R1r (DCcee) or R0r (Dccee). R0r was chosen as selection criteria because it is the most prevalent RH phenotype among Blacks [1,10,11], whereas R1r was selected aiming to identify individuals with r'S type I genotype, an important *RH* variant in SCD patient population. Group O criterion was established based on the following: 1) Most people of African ancestry in Brazil are of group O; and 2) Group O units can be used to transfuse patients of other blood types (A, B and AB) and, consequently, are more advantageous.

2.2. RH phenotyping

All selected donors were phenotyped for D, C, c, E, e through microplate hemaglutinnation method in an automated equipment (NEO, Immucor, Norcross, GA, EUA), according to the manufacturer's instructions.

2.3. DNA extraction

DNA was isolated using the Genomic PureLink kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Concentration and purity of the genomic DNA was checked through spectrophotometry (Nanodrop 1000, Wilmington, DE, USA). All DNA samples were diluted until a final concentration of 100 ng/uL for the genotyping assays.

2.4. FY*02 N.01 genotyping

Two hundred and fourteen donors were genotype for the c.−67 T > C polymorphism of the *FY*B* promoter region (*FY*02 N.01* allele) and classified as mutated/mutated (MM), mutated/wild type (MW) and wild-type/wild-type (WW). The RFLP genotyping protocol was described elsewhere [12].

2.5. RH genotyping

The direct sequencing of *RHCE* exons 1, 5, 6 and 7 was performed through the Sanger method for all included donors. These exons were selected because they contained the gene variations involved in the *RHCE* variants most commonly identified among SCD patients, as described in Table 1 of Supplementary Material [1,10,12,13].

Previously described gene-specific primers and PCR conditions were used for exon amplification [14–16]. PCR products were purified using ExoSAP (Applied Biosystems, Foster City, CA, USA) and prepared for sequencing in the ABI 3500xl equipment (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions.

A multiplex reaction designed to detect *RHD* variants was performed for all samples with the following *RHCE*: *RHCE*ceAR*, *RHCE*ceTI*, *RHCE*ceEK*, *RHCE*ceMO*, *RHCE*ceSM*, *RHCE*ceVS.03* and *RHCE*ceVS.05* [17]. All donors presenting the alleles *RHCE*ceVS.03* or *RHCE*ceVS.05* were checked for the presence of the *RHDIIIa-CE(4–7)-D* allele in *cis* through previously described assays [18]. *RHD* direct sequencing was performed for all cases that could not be resolved based on conventional molecular methods, as previously described [14,15].

2.6. Statistical analysis

The variable Number Needed to Screen (NNS) was calculated to express how many donors would have to be selected (based on a certain criteria) and *RH* genotyped to identify one individual with the desired predicted phenotype.

The groups MM, MW and WW were compared in terms of numbers of donors with clinically relevant predicted RH phenotype using the Chi-square test held on the SPSS software (20th version). A p value less than 0.05 was considered as significant.

3. Results

3.1. Donor recruitment

Two thousand and five hundred Brazilian donors were enrolled in the study. Of these, 217 blood donors fulfilled the inclusion criteria and were selected for RH genotyping. Of these, 117 (54 %) typed as R1r and 100 (46 %) typed as R0r. All donors self-declared as black. Two hundred and fourteen donors were genotyped for *FY*B* c.−67 T > C, as, in three cases, there was no DNA left for this assay.

3.2. RHCE variants

One hundred and seventy altered RHCE alleles were identified in the study (170/434 genotyped alleles, 39.1 %) (Table 1). Most variant *RHCE* listed in Table 1 were identified. One donor presented the variant *RHCE*ceSM*, that was not listed in Table 1 of Supplementary Material. The most frequent altered *RHCE* were *RHCE*ce.01* (55/434, 12.7 %), *RHCE*ceVS.01* (54/434, 12.4 %), *RHCE*ceVS.02* (23/434, 5.3 %) and *RHCE*ceAR* (15/434, 3.4 %) (Table 1). There were 91 RHCE alleles encoding hrB- phenotype (91/434 studied alleles, 20.9 %): *RHCE*ceVS.01* (54/434, 12.4 %), *RHCE*ceVS.02* (23/434, 5.3 %),

Table 1
RHCE alleles identified in the blood donor population selected using self-declared race and Rh phenotype.

Alleles	Number of alleles (%)	RHCE predicted phenotype
RHCE*ce	156 (36 %)	c+, e+
RHCE*Ce	108 (25 %)	C+, e+
RHCE*ce.01	55 (12.7 %)	weak e
RHCE*ceVS.01	54 (12.4 %)	partial c, partial e, V+, VS+, hrB-
RHCE*ceVS.02	23 (5.3 %)	partial c, partial e, V+, VS+, hrB-
RHCE*ceAR	15 (3.4 %)	partial c, partial e, V+^{W}, VS-, hrS-
RHCE*ceVS.05	9 (2.1 %)	partial e, V-, VS+, hrB-
RHCE*ceTI	5 (1.1 %)	partial c, partial e
RHCE*ceVS.03	3 (0.7 %)	partial c, partial e, V-, VS+, hrB-
RHCE*ceEK	2 (0.5 %)	partial c, partial e, hrS-
RHCE*ceSM	1 (0.2 %)	e+/-, hrS-, STEM+
RHCE*ceMO	1 (0.2 %)	partial c, partial e, hrS-, hrB-
RHCE*ceTI type2 like	1 (0.2 %)	partial e, V+, VS+, hrB-
RHCE*Ce.30	1 (0.2 %)	V+, VS+

*RHCE*ceVS.05* (9/434, 2.1 %), *RHCE*ceVS.03* (3/434, 0.7 %), *RHCE*ceMO* (1/434, 0.2 %) and *RHCE*ceTI type2-like* (1/434, 0.2 %). There were 19 alleles encoding hrS- phenotype (19/434, 4.37 %): *RHCE*ceAR* (15/434, 3.5 %), *RHCE*ceEK* (2/434, 0.5 %), *RHCE*ceMO* (1/434, 0.2 %) and *RHCE*ceSM* (1/434, 0.2 %).

Among the included donors, 140 (64.7 %) had at least one altered RHCE allele and 107 (49.3 %) had predicted variant RhCE phenotype (Table 2). Forty seven donors (47/217, 21.6 %) had a predicted clinically relevant phenotype (partial antigens or lack of high frequency antigens) and the prevalence of clinically relevant altered *RHCE* was 26.3 % (114 relevant alleles / 434 total genotyped alleles). Twelve donors (5.5 %) had predicted RhCE phenotype lacking either hrB (11 donors) or hrS (1 donor) (Table 2). The number needed to screen (NNS) to identify one hrB- and one hrS- donor using self-declared race and Rh phenotype as inclusion criteria was 19.7 and 217, respectively.

A summary of these results is displayed in Fig. 1 of Supplementary Material.

3.3. RHD variants in cis to altered RHCE

Thirty six donors (n = 72 alleles) were genotyped for *RHD*. The most frequently identified *RHD* were: *RHD*01* (31/72, 43 %), *RHD*DAR* (15/72, 20.8 %) and *RHD*DIIIa-CE(4–7)-D* (11/72, 15.3 %) (Table 3). The *RH* haplotypes present in the donors with altered *RHCE* (*RHCE*ceAR*, *RHCE*ceTI*, *RHCE*ceEK*, *RHCE*ceMO*, *RHCE*ceSM*, *RHCE*ceVS.03* e *RHCE*ceVS.05*) are shown in Table 4.

*RHD*DAR* was in *cis* to *RHCE*ceAR* in 15 alleles (15/434 genotyped alleles, 3.5 %). One donor (1/217 included donors, 0.4 %) had the haplotype *RHD*DAR / RHCE*ceAR* in homozygosity, predicting a partial D, c, e and hrS- phenotype. The *RHD*DIIIa-CE(4–7)-D*, responsible for the expression of partial C, was detected in 12 of 13 donors presenting the *RHCE*ceVS.03* and *RHCE*ceVS.05* alleles. A total of 8 donors (8/217, 3.7 %) had a predicted partial D phenotype.

In one case, *RHD*DV* was presumably in *cis* to *RHCE*ceTI type 2-like*, which is unusual.

3.4. Impact of Duffy null genotype as a selection criteria for the identification of RH variant blood donors

Two hundred and fourteen donors were genotyped for the *FY*02N.01* allele (c.−67 T > C GATA mutation of *FY*B*). The calculated allele frequency was 0.484 for the c.−67 T allele and 0.516 for the mutated c.-67C allele. The population was in Hardy-Weinberg equilibrium. There were 64 donors genotyped as mutated/mutated - MM (64/214, 30 %); 93 genotyped as mutated/wild-type – MW (93/214, 43 %) and 57 genotyped as wild-type/wild-type –WW (57/214, 27 %). The diversity and distribution of altered *RHCE* was similar between the MM, MW and WW groups (Table 5). Similarly, the frequency of individuals with altered *RHCE* genotype was homogeneous between the groups MM (33/64, 51.56 %), MW (47/93, 50.5 %) and WW (27/57, 47.37 %) (p = 0.89).

The frequency of individuals lacking the high prevalence antigens hrB or hrS differed significantly between the groups: 66.7 % were

Table 2
RHCE genotype of the blood donor population selected for the study using self-declared race and Rh phenotype.

RHCE genotype	Number of Donors (%)	Presumed phenotype	Rh phenotype
RHCE*Ce/*ce	57 (26.3 %)	C+, c+, e+	R_1r
RHCE*Ce/*ce.01	21 (9.6 %)	C+, c+, e+	
RHCE*ce/*ce	20 (9.3 %)	c+, e+	R_0r
RHCE*ceVS.01/*ce	19 (8.7 %)	V+, VS+	R_0r
RHCE*ceVS.01/*Ce	19 (8.7 %)	partial c, V+, VS+	R_1r
RHCE*ceVS.02/*ce	15 (6.9 %)	V+, VS+	R_0r
RHCE*ce.01/*ce	10 (4.6 %)	c+, e+	
RHCE*ce.01/*ce.01	8 (3.6 %)	weak e	
RHCE*ceAR/*ce	7 (3.2 %)	V+^{W}	
RHCE*ceVS.02/*Ce	4 (1.9 %)	partial c, V+, VS+	R_1r
RHCE*ceVS.01/*ceVS.01	4 (1.9 %)	partial c, partial e, V+, VS+, hr^{B}-	R_0r
RHCE*ceVS.01/*ceVS.05	3 (1.3 %)	partial C*, partial e, V + VS+, hr^{B}-	R_1r
RHCE*ceAR/*ce.01	3 (1.3 %)	variant e, V+^{W}	R_0r
RHCE*ceVS.01/*ceVS.03	2 (0.9 %)	partial C*, partial c, partial e, V+, VS+, hr^{B}-	R_1r
RHCE*ceVS.05/*ce	2 (0.9.%)	partial C*, V-, VS+	
RHCE*ceTI/*Ce	2 (0.9 %)	partial c	
RHCE*ceTI/*ce.01	2 (0.9 %)	variant e	R_0r
RHCE*ceVS.02/*ce.01	2 (0.9 %)	variant e, V+,VS+	
RHCE*ceVS.02/*ceVS.01	2 (0.9 %)	partial c, partial e, V+, VS+, hr^{B}-	
RHCE*ceVS.05/*Ce	2 (0.9 %)	partial c, V-, VS+	R_1r
RHCE*ceAR/*Ce	2 (0.9 %)	partial c, V+^{W}	
RHCE*ceEK/*Ce	1 (0.5 %)	partial c	
RHCE*ceVS.03/*ce	1 (0.5 %)	partial C*, V-, VS+	
RHCE*ceEK/*ce	1 (0.5 %)	c+, e+	R_0r
RHCE*ceTI/*ce	1 (0.5 %)	c+, e+	
RHCE*ceAR/*ceAR	1 (0.5 %)	partial c, partial e, V+^{W}, VS-, hr^{S}-	
RHCE*ceAR/*ceVS.01	1 (0.5 %)	partial c, partial e, V+,VS+	
RHCE*ceSM/*ce	1 (0.5 %)	STEM+^{W}	
RHCE*ceTI type 2 like/*ce	1 (0.5 %)	V+, VS+	
RHCE*ceMO/*ce.01	1 (0.5 %)	variant e	
RHCE*ceVS.05/*Ce.30	1 (0.5 %)	partial c, V+, VS+	R_1r
RHCE*ceVS.05/*ce	1 (0.5 %)	V-, VS+	R_0r

* Altered *RHCE* in *cis* to *RHD*DIIIa-CE(4–7)-D*.

Table 3
RHD alleles identified in the studied blood donor population.

Alleles	Number of alleles (n/%)	Predicted Phenotype
RHD*01	31 (43 %)	D
RHD*DAR	15 (20.8 %)	partial D, DAK+
RHD*DIIIa-CE(4–7)-D	11 (15.3 %)	D-, C+
deleted RHD	8 (11.1 %)	D-
RHD*DIVa	3 (4.2 %)	partial D, Goa+
RHD*DAU0	1 (1.4 %)	partial D
RHD*DIIIa	1 (1.4 %)	partial D, DAK+
RHD*DV	1 (1.4 %)	partial D
RHD*DOL1	1 (1.4 %)	partial D

Table 4
Altered *RH* haplotypes identified in the study.

Number of Samples	*RH* Haplotypes			
	RHD		*RHCE*	
	Allele1	*Allele2*	*Allele1*	***Allele2***
5	*RHD*DAR*	*RHD*Deletado*	*RHCE*ceAR*	*RHCE*ce*
3	*RHD*DAR*	*RHD*01*	*RHCE*ceAR*	*RHCE*ce.01*
3	*RHD*DIIIa-CE (4–7)-D*	*RHD*01*	*RHCE*ceVS.05*	*RHCE*ceVS.01*
2	*RHD*DAR*	*RHD*01*	*RHCE*ceAR*	*RHCE*ce*
2	*RHD*DAR*	*RHD*01*	*RHCE*ceAR*	*RHCE*Ce*
2	*RHD*DIIIa-CE (4–7)-D*	*RHD*01*	*RHCE*ceVS.03*	*RHCE*ceVS.01*
2	*RHD*DIIIa-CE (4–7)-D*	*RHD*01*	*RHCE*ceVS.05*	*RHCE*ce*
2	*RHD*DIIIa-CE (4–7)-D*	*RHD*01*	*RHCE*ceVS.05*	*RHCE*Ce*
1	*RHD*DIIIa-CE (4–7)-D*	*RHD*01*	*RHCE*ceVS.05*	*RHCE*Ce.30*
1	*RHD*DIIIa*	*RHD*01*	*RHCE*ceVS.05*	*RHCE*ce*
1	*RHD*DIIIa-CE (4–7)-D*	*RHD*01*	*RHCE*ceVS.03*	*RHCE*ce*
1	*RHD*DAR*	*RHD*DAR*	*RHCE*ceAR*	*RHCE*ceAR*
1	*RHD*DAR*	*RHD*01*	*RHCE*ceAR*	*RHCE*ceVS.01*
1	*RHD*01*	*RHD*01*	*RHCE*ceEK*	*RHCE*ce*
1	*RHD*01*	*RHD*01*	*RHCE*ceEK*	*RHCE*Ce*
1	*RHD*DIVa*	*RHD*01*	*RHCE*ceTI*	*RHCE*Ce*
1	*RHD*01*	*RHD*01*	*RHCE*ceTI*	*RHCE*Ce*
1	*RHD*DIVa*	*RHD*01*	*RHCE*ceTI*	*RHCE*ce*
1	*RHD*DIVa*	*RHD*01*	*RHCE*ceTI*	*RHCE*ce.01*
1	*RHD*01*	*RHD*01*	*RHCE*ceTI*	*RHCE*ce.01*
1	*RHD*DV*	*RHD*Deleted*	*RHCE*ceTI type 2 like*	*RHCE*ce*
1	*RHD*DAU0*	*RHD*Deleted*	*RHCE*ceMO*	*RHCE*ce.01*
1	*RHD*DOL1*	*RHD*Deleted*	*RHCE*ceSM*	*RHCE*ce.01*

identified in the MM group (8/12); 25 % were identified in the MW group (3/12) and 8.3 % were identified in the WW group (1/12) (p = 0.0076).

The number of donors needed to screen (NNS) to identify one individual with presumed clinically relevant RhCE phenotype using self-declared race, Rh phenotype and *FYB* c.−67 T > C genotype as selection criteria was 3.5 for the MM group, 4.8 for the MW group and 5.7 for the WW group. However, the NNS to identify one donor lacking a high frequency antigen (hrB or hrS) was 8 in the MM group, 31 in the MW group and 57 in the WW group. The proportion of samples in which the alleles encoding the lack of high frequency antigen (*RHCE*ceVS.01, RHCE.ceVS.02, RHCE*ceVS.03, RHCE*ceVS.05* and *RHCE*ceAR*) was in *trans* to either a non-altered *RHCE* or to an altered allele predicting the presence of high frequency antigens was higher in the groups MW and WW (92.7 % and 95.7 %) in comparison to MM group (70.4 %) (p = 0.01).

A summary of these results is displayed in Fig. 2 of Supplementary Material.

4. Discussion

The present study aimed to standardize a strategy to identify *RH* variants among a highly mixed donor population. It was demonstrated that: 1) By selecting donors based on self-declared race and Rh phenotype (R1r or R0r), 64.7 % (140/217) had at least one altered *RHCE* and 49.3 % (107/217) had predicted variant RhCE phenotype; 2) The number needed to screen (NNS) to identify a donor with a clinically relevant RhCE phenotype (partial antigens and/or lack of high incidence antigens) was 4.1; 3) The inclusion of the Duffy null genotype or Fy(a-b-) phenotype as selection criteria together with self-declared race and Rh phenotype increased the chances of identifying donors lacking high frequency antigens (hrB and hrS); 4) The most prevalent *RHCE* variants identified among the selected donor population were: *RHCE*ce.01* (12.7 %), *RHCE*ceVS.01* (12.4 %), *RHCE*ceVS.02* (5.3 %) and *RHCE*ceAR* (3.4 %).

Previous studies have evaluated the frequency of *RH* variations among blood donors selected based on the self-declared race [11,13]. The largest studies did not comprise racially mixed population such as Brazilian, in which there may be a significant error associated with self-referred race classification. In the present study, self-declared race and Rh phenotype were used as selection criteria for the screening of RHCE altered alleles. Our results show that selecting donors based on these criteria is effective for the identification of *RH* variants in the scenario of a mixed population, as approximately one quarter (21.6 %) of the included black individuals (1.9 % of total pool of 2500 blood donors) had predicted partial Rh antigens and/or lack of high frequency antigens and could be used to meet the needs of sensitized patients. The rate of altered RHCE alleles identified in the present study (64.7 %) was superior to that previously described for both African-American and African-Brazilian donors, probably reflecting the impact of the Rh phenotype as inclusion criteria [11,13,19]. Comparing our results with previous studies also focusing on mixed populations, the prevalence of predicted clinically relevant altered Rh phenotype (partial antigens and/or lack of high frequency antigens) was significantly higher (21.6 % versus 1.4–6.6%) [13,20].

This study was the first to evaluate the impact of including the Duffy null genotype (*FY*02 N.01/FY*02 N.01*) as criteria for selecting donors for the search of predicted Rh variant phenotypes. The *FY*02 N.01* allele is an ancestry-informative marker extremely prevalent among people of African descent. Its surrogate marker is the Fy(a-b-) phenotype, which can be easily accessed in the immunohematological routine. Our results showed that the prevalence of individuals with predicted lack of high frequency Rh antigens was eight times higher in the group presenting the Duffy null genotype in comparison to the non-mutated group of donors. As so, if the aim of the search for *RH* variant donors is to identify individuals whose genotype predicts the lack of Rh high frequency antigens (in the studied population mostly represented by *RHCE*ceVS.01, RHCE*ceVS.02, RHCE*ceAR, RHCE*ceVS.05, RHCE*ceVS.03* and *RHCE*ceMO* in homozygosity or compound heterozygosity), then selecting donors based either on the Duffy null genotype or on the Fy(a-b-) phenotype, besides using the self-declared race and Rh phenotype criteria, seems advantageous. Considering that antibodies directed to RH high frequency antigen are commonly involved in situations in which *RH* variant-matched units are required for transfusion, then the Duffy null genotype represents a cost-effective criterion for selecting donors for *RH* genotyping in mixed population.

In the present study, the initial option was to select donors stemming from racially mixed population based on pre-defined criteria (black as self-declared race and Rh phenotype) and to genotype, through direct sequencing, *RHCE* exons 1, 5, 6 and 7 to identify and accurately classify the variants most commonly found in our population of alloimmunized SCD patients. However, for screening purposes, the sequencing strategy is not very attractive. Based on the identified clinically relevant *RHCE*, a more reasonable strategy would be to test the selected donors for the c.733C > G polymorphism (using conventional molecular methods) in order to identify the homozygous individuals and, then, expand the *RH* genotyping using either DNA-array methods or direct sequencing of *RHCE* exons 1, 5 and 7. If this algorithm was applied in the present cohort of donors (n = 217), 13 individuals would have been selected for extended *RH* genotyping, of whom 12 would have predicted lack of high frequency RH antigens. If only Fy(a-b-) donors were included (n = 64), 9 would present the c.733C > G polymorphism in homozygosity and, of these, 8 would eventually be classified as lacking high frequency Rh antigens. The decision to perform genotyping or phenotyping to access the Duffy null genotype depends on the laboratory workflow. If a certain amount of donors are regularly phenotyped for both Fya and Fyb, then phenotyping is a best option to determine the Duffy null status. However, if

Table 5
Distribution and diversity of RHCE alleles and genotype identified in the selected donor population according to the *FY*B* c.−67 T > C genotype.

RHCE genotype (% of individuals)

MM group (n = 64 donors)		*MW group (n = 93 donors)*		*WW group (n = 57 donors)*	
RHCE*Ce/*ce	12 (18.8 %)	***RHCE*Ce/*ce***	26 (27.9 %)	***RHCE*Ce/*ce***	18 (31.6 %)
RHCE*ce/*ce	7 (10.9 %)	***RHCE*ceVS.01/*Ce***	10 (10.8 %)	***RHCE*ceVS.01/*Ce***	7 (12.3 %)
RHCE*Ce/*ce.01	7 (10.9 %)	***RHCE*ceVS.01/*ce***	10 (10.8 %)	***RHCE*ceVS.01/*ce***	6 (10.5 %)
RHCE*ceVS.02/*ce	7 (10.9 %)	***RHCE*Ce/*ce.01***	9 (9.7 %)	***RHCE*ce/*ce***	5 (8.8 %)
RHCE*ce.01/*ce	5 (7.8 %)	***RHCE*ce/*ce***	7 (7.5 %)	***RHCE*Ce/ce.01***	4 (7 %)
RHCE*ceVS.01/*ce	3 (4.7 %)	***RHCE*ceVS.02/*ce***	6 (6.4 %)	***RHCE*ceAR/*ce***	3 (5.3 %)
RHCE*ce.01/*ce.01	2 (3.1 %)	***RHCE*ce.01/*ce.01***	4 (4.3 %)	***RHCE*ce/ce.01***	2 (3.5 %)
RHCE*ceVS.01/*ceVS.03	2 (3.1 %)	***RHCE*ceVS.02/*Ce***	3 (3.2 %)	***RHCE*ce.01/*ce.01***	2 (3.5 %)
RHCE*ceVS.01/*ceVS.01	2 (3.1 %)	***RHCE*ce/*ce.01***	3 (3.2 %)	***RHCE*ceAR/*ce.01***	2 (3.5 %)
RHCE*ceAR/*ce	2 (3.1 %)	***RHCE*ceAR/*ce***	2 (2.1 %)	***RHCE*ceVS.02/*ce***	2 (3.5 %)
RHCE*ceVS.01/*Ce	2 (3.1 %)	***RHCE*ceVS.01/*ceVS.01****	2 (2.1 %)	***RHCE*ceTI/*ce***	1 (1.75 %)
RHCE*ceTI/*Ce	2 (3.1 %)	***RHCE*ceVS.02/*ce.01***	2 (2.1 %)	***RHCE*ceTI/*ce.01***	1 (1.75 %)
RHCE*ceVS.05/*ce	2 (3.1 %)	***RHCE*ceEK/*ce***	1 (1.1 %)	***RHCE*ceVS.01/*ceVS.05****	1 (1.75 %)
RHCE*ceVS.02/*ceVS.01	2 (3.1%)	***RHCE*ceVS.05/*ce***	1 (1.1%)	***RHCE*ceMO/*ce.01***	1 (1.75%)
RHCE*ceAR/*ceVS.01	1 (1.6 %)	***RHCE*ceSM/*ce***	1 (1.1 %)	***RHCE*ceAR/*Ce***	1 (1.75 %)
RHCE*ceAR/*ceAR	1 (1.6 %)	***RHCE*ceVS.03/*ce***	1 (1.1 %)	***RHCE*ceVS.02/*Ce***	1 (1.75 %)
RHCE*ceEK/*Ce	1 (1.6 %)	***RHCE*ceVS.01/*ceVS.05****	1 (1.1 %)		
RHCE*ceVS.01/*ceVS.05	1 (1.6 %)	***RHCE*ceTI type 2 like/*ce.01***	1 (1.1 %)		
RHCE*ceTI/*ce.01	1 (1.6 %)	***RHCE*ceAR/*Ce***	1 (1.1 %)		
RHCE*ceVS.05/*Ce	1 (1.6 %)	***RHCE*ceVS.05/*Ce***	1 (1.1 %)		
RHCE*ceVS.05/*Ce.30	1 (1.6 %)	***RHCE*ceAR/*ce.01***	1 (1.1 %)		

RHCE alleles (% of alleles)

MM group (n = 128 alleles)		*MW group (n = 186 alleles)*		*WW group (n = 114 alleles)*	
RHCE*ce	45 (35.2 %)	***RHCE*ce***	66 (35.5 %)	***RHCE*ce***	42 (36.8 %)
RHCE*Ce	25 (19.5 %)	***RHCE*Ce***	50 (26.9 %)	***RHCE*Ce***	31 (27.2 %)
RHCE*ce.01	17 (13.3 %)	***RHCE*ceVS.01***	25 (13.4 %)	***RHCE*ce.01***	14 (12.3 %)
RHCE*ceVS.01	15 (11.7 %)	***RHCE*ce.01***	23 (12.4 %)	***RHCE*ceVS.01***	14 (12.3 %)
RHCE*ceVS.02	9 (7 %)	***RHCE*ceVS.02***	11 (6 %)	***RHCE*ceAR***	6 (5.3 %)
RHCE*ceVS.05	5 (3.9 %)	***RHCE*ceAR***	4 (2.2 %)	***RHCE*ceVS.02***	3 (2.6 %)
RHCE*ceAR	5 (3.9 %)	***RHCE*ceVS.05***	3 (1.6 %)	***RHCE*ceTI***	2 (1.7 %)
RHCE*ceTI	3 (2.3 %)	***RHCE*ceEK***	1 (0.5 %)	***RHCE*ceVS.05***	1 (0.9 %)
RHCE*ceVS.03	2 (1.6 %)	***RHCE*ceVS.03***	1 (0.5 %)	***RHCE*ceMO***	1 (0.9 %)
RHCE*ceEK	1 (0.8 %)	***RHCE*ceSM***	1 (0.5 %)		
RHCE*Ce.30	1 (0.8 %)	***RHCE*ceTI type 2 like***	1 (0.5 %)		

phenotyping is not routinely performed, then genotyping using conventional molecular methods represents a more cost-effective strategy. The suggested workflow for screening for blood donors with clinically relevant *RH* variants in a mixed population is described in Fig. 3 of Supplementary Material.

Finally, in this study, the distribution of *RHCE* variations among self-declared black blood donors from a population of mixed origin could be determined. The most common altered *RHCE* alleles were (% of total altered alleles): *RHCE*ce.01* (12.7 %), *RHCE*ceVS.01* (12.4 %), *RHCE*ceVS.02* (5.3 %), *RHCE*ceAR* (3.4 %) and *RHCE*ceVS.05* (2.1 %). The high frequency of both *RHCE*ce.01* and *RHCE*ceVS.01* alleles was in accordance with previous studies focusing on African-American blood donors [10,11]. In our studied African-Brazilian donor population, though, the prevalence of *RHCE*ceAR* was much higher than that reported for African-American donors. Previous Brazilian cohorts of blood donors have also reported a higher frequency of *RHCE*ceAR*, suggesting this allele to be relatively frequent in our population [13,20].

In conclusion, selecting blood donors based on self-declared race, Rh phenotype and Duffy null genotype, or Fy(a-b-) phenotype, is an effective alternative to screen for *RH* variants in a mixed population. This strategy might be helpful for centers transfusing sickle cell disease patients in which the donor population is racially mixed and *RH*-matched transfusions may be required.

Declaration of Competing Interest

None.

Acknowledgements

Almeida FAA, Dezan MR, Oliveira VB, Alencar CS and Luz FL: performed the experiments; Krieger JE, Pereira AC, Sabino EC, Rocha V, Mendrone-Júnior A and Dinardo CL: designed the study and oversaw the experiments; Almeida FAA, Pereira AC and Dinardo CL: wrote the manuscript.

This research was funded by Fundação Pró-Sangue Hemocentro de São Paulo, Brazil (no grant number).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.transci.2020.102720.

References

[1] Reid ME, Halter Hipsky C, Hue-Roye K, Hoppe C. Genomic analyses of RH alleles to improve transfusion therapy in patients with sickle cell disease. Blood Cells Mol Dis 2014;52:195–202.
[2] Flegel WA. The genetics of the Rhesus blood group system. Blood Transfus 2007;5:50–7.
[3] Sassi A, Ouchari M, Houissa B, Romdhane H, Abdelkefi S, Chakroun T, et al. RHD genotyping and its implication in transfusion practice. Transfus Apher Sci 2014;51:59–63.
[4] Flegel WA. Molecular genetics and clinical applications for RH. Transfus Apher Sci 2011;44:81–91.
[5] Rosse WF, Gallagher D, Kinney TR, Castro O, Dosik H, Moohr J, et al. Transfusion and alloimmunization in sickle cell disease. The cooperative study of sickle cell disease. Blood 1990;76:1431–7.
[6] Sakhalkar VS, Roberts K, Hawthorne LM, McCaskill DM, Veillon DM, Caldito GC,

et al. Allosensitization in patients receiving multiple blood transfusions. Ann N Y Acad Sci 2005;1054:495–9.
[7] Hendrickson JE, Hod EA, Perry JR, Ghosh S, Chappa P, Adisa O, et al. Alloimmunization to transfused HOD red blood cells is not increased in mice with sickle cell disease. Transfusion 2012;52:231–40.
[8] Smith NH, Hod EA, Spitalnik SL, Zimring JC, Hendrickson JE. Transfusion in the absence of inflammation induces antigen-specific tolerance to murine RBCs. Blood 2012;119:1566–9.
[9] Dezan VBO JVSB MR, Rodrigues V, Solano JH, Gomes FC, Bonifácio SL, Levi JE, et al. Effectiveness of a red cell antigen-matching transfusion protocol in sickle cell disease patients. ISBT Sci Ser 2016;11:132–9.
[10] Chou ST, Jackson T, Vege S, Smith-Whitley K, Friedman DF, Westhoff CM. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors. Blood 2013;122:1062–71.
[11] Chou ST, Evans P, Vege S, Coleman SL, Friedman DF, Keller M, et al. RH genotype matching for transfusion support in sickle cell disease. Blood 2018;132:1198–207.
[12] Dinardo CL, Kerbauy MN, Santos TC, Lima WM, Dezan MR, Oliveira VB, et al. Duffy null genotype or Fy(a-b-) phenotype are more accurate than self-declared race for diagnosing benign ethnic neutropenia in Brazilian population. Int J Lab Hematol 2017;39:e144–6.
[13] Gaspardi AC, Sippert EA, De Macedo MD, Pellegrino Jr. J, Costa FF, Castilho L. Clinically relevant RHD-CE genotypes in patients with sickle cell disease and in African Brazilian donors. Blood Transfus 2016;14:449–54.
[14] Dezan MR, Guardalini LGO, Pessoa E, Ribeiro IH, Oliveira VB, Luz F, et al. Evaluation of the applicability and effectiveness of a molecular strategy for identifying weak D and DEL phenotype among D- blood donors of mixed origin exhibiting high frequency of RHD*Psi. Transfusion 2018;58:317–22.
[15] Legler TJ, Maas JH, Kohler M, Wagner T, Daniels GL, Perco P, et al. RHD sequencing: a new tool for decision making on transfusion therapy and provision of Rh prophylaxis. Transfus Med 2001;11:383–8.
[16] Wagner FF, Gassner C, Muller TH, Schonitzer D, Schunter F, Flegel WA. Three molecular structures cause rhesus D category VI phenotypes with distinct immunohematologic features. Blood 1998;91:2157–68.
[17] Maaskant-van Wijk PA, Faas BH, de Ruijter JA, Overbeeke MA, von dem Borne AE, van Rhenen DJ, et al. Genotyping of RHD by multiplex polymerase chain reaction analysis of six RHD-specific exons. Transfusion 1998;38:1015–21.
[18] Tax MG, van der Schoot CE, van Doorn R, Douglas-Berger L, van Rhenen DJ, Maaskant-vanWijk PA. RHC and RHc genotyping in different ethnic groups. Transfusion 2002;42:634–44.
[19] Prisco Arnoni C, Guilhem Muniz J, de Paula Vendrame TA, de Medeiros Person R, Roche Moreira Latini F, Castilho L. RHCE variants inherited with altered RHD alleles in Brazilian blood donors. Transfus Med 2016;26:285–90.
[20] Cruz BR, de Souza Silva TC, de Souza Castro B, Chiba AK, Moritz E, Braga JP, et al. Molecular matching for patients with haematological diseases expressing altered RHD-RHCE genotypes. Vox Sang 2019;114(6):605–15.