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RHD VARIANTS IN POLISH BLOOD DONORS ROUTINELY TYPED AS D NEGATIVE

Agnieszka Orzińska, **Katarzyna Guz**, **Helene Polin**, **Monika Pelc-Kłopotowska**, **Justyna Bednarz**, **Agata Gieleżyńska**, **Beata Śliwa**, **Małgorzata Kowalewska**, **Elżbieta Pawłowska**, **Bogusława Włodarczyk**, **Małgorzata Malaga**, **Alicja Żmudzin**, **Magdalena Krzemienowska**, **Kshitij Srivastava**, **Bogumiła Michalewska**, **Christian Gabriel**, **Willy A Flegel**, and **Ewa Brojer**

Department of Immunohematology and Immunology of Transfusion Medicine, Institute of Haematology and Blood Transfusion, Warsaw, Poland; Regional Blood Transfusion Centers in Warsaw, Kraków, Gdańsk, Racibórz, Kalisz, Kielce & Radom, Poland; Red Cross Transfusion Centre of Upper Austria, Linz, Austria, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Abstract

BACKGROUND—Blood donors exhibiting a weak D or DEL phenotypical expression may be mistyped D negative by standard serology hence permitting incompatible transfusion to D negative recipients.

Molecular methods may overcome these technical limits. Our aim was to estimate the frequency of *RHD* alleles among the apparently D negative Polish donor population and to characterize its molecular background.

STUDY DESIGN AND METHODS—Plasma pools collected from 31,200 consecutive Polish donors typed as D negative were tested by real-time PCR for the presence of *RHD* specific markers located in the intron 4, exons 7 and 10. *RHD* positive individuals were characterized by PCR or cDNA sequencing and serology.

RESULTS—Plasma cross-pool strategy revealed 63 *RHD* positive donors harboring *RHD***01N.03*(n=17), *RHD***15*(n=12), *RHD***11*(n=7), *RHD***DEL8*(n=3), *RHD***01W.2*(n=3), *RHD-CE(10)* (n=3), *RHD***01W.3*, *RHD***01W.9*, *RHD***01N.05*, *RHD***01N.07*, *RHD***01N.23*, *RHD*(*IVS1-29G>C*) and two novel alleles: *RHD**(*767C>G)*(n=3), *RHD***(1029C>A)*. Among 47 cases available for serology, 27 were shown to express the D antigen

CONCLUSION—1/ Plasma cross-pool strategy is a reliable and cost-effective tool for *RHD* screening. 2/ 0.2% of D negative Polish donors carry some fragments of the *RHD* gene; all of them were C or E positive. 3/ Almost 60% of the detected *RHD* alleles may be potentially immunogenic when transfused to a D negative recipient.

Address reprint requests to: Agnieszka Orzińska, Katarzyna Guz Institute of Haematology and Blood Transfusion, 5st Chocimska Street, 00 957 Warsaw, Poland; aorzinska@ihit.waw.pl, kguz@ihit.waw.pl. Agnieszka Orzińska: address: Institute of Haematology and Blood Transfusion, 5st Chocimska Street, 00-957 Warsaw, Poland; tel/fax: +48 223496611; aorzinska@ihit.waw.pl.

Conflict of interest

The other authors declare that they have no conflicts of interest relevant to this publication.

Keywords

RHD alleles; RhD negative blood donors; plasma pools; real-time PCR

INTRODUCTION

In European blood donors, the D negative phenotype is mainly results from a complete deletion of the *RHD* gene. However, in some individuals the *RHD* gene or its fragments are present while the RhD protein is either not expressed at all, has a lower level of expression at the red blood cell (RBC) membrane or lacks some epitopes. [1–4]

Standard serologic methods cannot detect D variants with a reduced amount of D antigen. Red blood cells (RBCs) of patients harbouring weak D types were described to stimulate anti-D immunization after transfusion to D negative (neg) patients.[5–7] Dneg patients transfused with D positive (pos) blood products routinely mistyped as Dneg may be at risk of anti-D immunization.[5] DEL variants expressing an extremely low density of RhD protein on the cell surface remain undiscovered even by sensitive serologic tests (with the exception of adsorption-elution (ads/el) technique) were reported to induce anti-D production in Dneg recipients.[6–10]

Up to date, more than 80 weak D and 17 DEL types have been described.[3–4] To prevent immunization events, donors with a potentially immunogenic D antigen encoded by an aberrant *RHD* allele should always be typed Dpos. However, a high variety of known *RHD* gene formations as well as the need for routine large-scale blood donor testing makes the serologic identification of weak D alleles difficult and practically impossible in case of DELs. Better knowledge of *RHD* background and availability of molecular techniques has initiated a new stage of typing strategies for blood donors.[11–15] Various strategies for *RHD* screening among apparently Dneg donors have been introduced to identify potentially immunogenic RhD variants, hence classify them as Dpos. DNA based *RHD* typing is performed once in a lifetime. Moreover, molecular screening has provided numerous details on *RHD* background in different populations.[16–24] The frequency and composition of *RHD* alleles among apparently Dneg Polish donors is unknown. The prevalence of other markers encoding platelet or granulocyte antigens in Polish individuals indicates that our population differs slightly from Central or Western Europeans.[25] In this study 31,200 consecutive Dneg blood donors from 7 regions of Poland were analyzed for the presence of *RHD* markers. Our aim was to determine the background of RhD negativity in the Polish population and to analyze the aberrant *RHD* alleles in seemingly Dneg blood donors. To reduce costs, we introduced a mini-pool testing strategy, routinely applied for screening of blood donors for viral nucleic acids.[26]

Firstly, we present the *RHD* background of our local population; secondly, we will create a database of D variant carriers in our country to be able to estimate the real risk of D negative immunization following DEL/weak D transfusion to D negative patient in the future.

MATERIAL AND METHODS

Blood sample collection

Blood was collected from 31,200 consecutive Dneg blood donors categorized as Dneg in routine serologic typing performed in 7 Polish Regional Blood Transfusion Centers (RBTC) (Warszawa, Racibórz, Kalisz, Radom, Kraków, Kielce, Gdańsk). According to Polish regulations, the D antigen of blood donors is determined by testing of two separate samples using two different monoclonal anti-D clones for each test. Three of the seven RBTCs, which took part in this survey, used the automated DiaMed/BioRad (Cressier, Switzerland) system (microplate and/or microcolumn techniques) with monoclonal anti-D of ESD-1M, 175-2, LHM50/3 LDM1) and RUM-1 clones or Immucor Gamma (Rodermark, Germany) Galileo system by Capture technique with monoclonal anti-D of D175, D415, RUM-1. In the other 4 centers, D antigen was typed manually using a combination of two techniques: microcolumn DiaMed/BioRad (anti-D clones as above), tube NaCl and indirect antiglobulin test (IAT) by anti-D clones: RUM-1 and TH28/MS26.

Blood samples were collected into EDTA vacutainer tubes with gel-barrier and centrifuged within 4 hours according to manufacturer's protocol to separate plasma from cell fractions. The whole blood samples of identified *RHD*pos donors were collected into EDTA vacutainer tubes. The tubes were sent to the Institute of Hematology and Transfusion Medicine (IHTM) in Warsaw and stored at −20°C until DNA extraction.

Pooling strategy and validation of *RHD* detection

48 plasma samples from D negative blood donors were pooled before DNA isolation and archive plates with all of them were prepared for further analysis using a robotic sample processor (Genesis RSP200, Tecan; Hombrechtikon, Switzerland) and stored at −80°C. Two 48-pools were combined manually to obtain one 96-sample plasma pool. If *RHD* gene was detected in the DNA isolated from 96 plasma pool, the 14 crosspools were prepared by pooling plasma samples from the archive plate (from 8 rows and 6 columns separately) using a workstation. Then DNA from these crosspools was tested for *RHD* (Fig. 1). The identification of *RHD*pos sample was performed with computer software (Pool Mangment System, Tecan Software, Hannover, Germany).

12 validation experiments were performed, which was spiked with plasma from one D positive donor, in a pool of 95 *RHD*neg plasma samples.

DNA extraction

DNA was extracted automatically by NucliSens easyMAG (bioMerieux, Marcy I'Etoile, France) using: 1 ml of plasma from either 96- or 48-pool; 500μL of plasma from the crosspool or 200μL of plasma from the archive plate in case of the identified *RHD*pos donor. The 60μL of buffer was used for DNA elution. Genomic DNA from *RHD*pos donors was isolated from 200μL of whole blood using NucleoSpin Blood Kit (Marcheney Nagle GmbH, Duren, Germany).

Real-time PCR for *RHD* variants

The presence of the *RHD* gene was analyzed by real-time PCR with sequence-specific primers and probes for three regions of the gene: exon 7, 10 and intron 4 (ABI Prism 7700, Applied Biosystems, Brauchburg, NJ) as described previously.[27] Firstly, DNA isolated from plasma pools of 96 RhDneg donors was tested. If any fragment of *RHD* gene was detected, DNA from two 48 pools was examined separately. All samples from the *RHD*+ archive plate were analyzed by cross-pooling strategy: 14 crosspools were tested for the *RHD* gene. *RHD*pos plasma sample was identified at the intersection of *RHD*pos crosspools on the archive plate (Fig.1). Finally, plasma DNA of individual *RHD*pos donor from the archive plate was retested to confirm the presence of the *RHD* gene.

RHD allele identification

DNA from the whole blood sample of *RHD*pos donors were genotyped for D variant by SSP-PCR using tests: RBC-READY GENE CDE, weak D, D AddOn (INNO-Train, Diagnostic GmbH, Kronberg/Tauns, Germany).

Nucleotide sequencing of *RHD* exons 1–10 was performed for 19 samples as previously described.[15] In 3 samples with *RHD-CE* hybrid genes, cDNA sequencing was conducted by applying: forward primer 5'-CACAGGATGAGCTCTAAGTAC-3' specific for *RHD/CE;* each reverse primer 5'-TAAATGGTGAGATTCTCCTC-3' specific for *RHD* and 5'-CTGTCTCTGACCTTGTTTCATTATAC-3' specific for *RHCE*.

Serological methods of RhD reevaluation of *RHD*pos donors at IHTM

The red blood cells of *RHD*pos blood donors tested as D negative in the routine screening at RBTC were reevaluated at IHTM by IAT with anti-D IgG commercial reagents other than those used at particular RBTC: (11B7IgG+BS232 IgM (Biotest, Dreieich, Germany), IgM RUM-1 (Immucor Gamma), BS-221/H41 D175 +D415IgG+IgM (Immucor Gamma, Rodermark, Germany), LDM3 IgM+ ESD1IgG, LDM1 IgM , LDM1IgM+ESD1M IgG (Alba Bioscience, Edinburgh, United Kingdom). If the reaction with any of these reagents was positive the sample was tested with a set of monoclonal anti-D for D category (Advanced Partial RhD typing kit, AlbaBioscience). If all serological tests were negative, ads/el method with human polyclonal anti-D IgG RhD (titer > 128) was applied.

RESULTS

During validation experiments, the *RHD*pos samples were determined in all cases and the cross-pooling strategy combined with real-time PCR detection of the *RHD* gene led to the identification the Dpos donor.

In 5 out of 325 96-plasma pools from Dneg donors, at least one *RHD* specific marker was present. After separating positive 96-pools into two 48-pools, *RHD* positive signals were detected in 63/118 48-pools and the protocol of cross-pooling was performed. It revealed 63/31,200 Dneg donors carrying parts of the *RHD* gene (Tab. 1).

Table 2 presents detailed results of serological and genetic tests performed at IHTM for *RHD*pos samples using the whole blood recollected from blood donors by 7 RBTCs. In our

donors' cohort, 15 different *RHD* alleles were determined with D antigen expression in 8 variants. In 6 cases of *RHD* alleles present in 10 individuals, a commercially available kit for *RHD* genotyping gave no satisfying result until the aberrant variant was evident in further sequencing.

19 Dneg donors were found positive only for exon 10 of the *RHD* gene (30% of all *RHD* detected donors). Further molecular analysis of whole blood allowed to identify three types of the *RHD-CE-D* hybrid gene: *RHD01N*03, *05, *07* in these cases. Serological retesting of 20 available samples confirmed the complete lack of D expression on the RBCs surface.

None of the donors was found positive only for exon 7 or intron 4 of the *RHD* gene. In all cases, except the above mentioned hybrid *RHD-CE-D* variants, both *RHD* fragments were detected in parallel. Among them, we identified 3 donors that were negative for exon 10. Further exon-screening by RBC-READY GENE CDE and by sequencing confirmed that only the exon 10 of the *RHD* was missing in these samples. cDNA sequencing confirmed the *RHD-CE(10)* hybrid. The presence of the D antigen was demonstrated by ads/el technique in those samples.

The remaining 41 samples gave *RHD*pos real-time PCR signal for all three tested fragments of the gene. After whole blood retesting by molecular biology and serology they were classified into 3 groups of *RHD* alleles. In the first group (17 donors), a positive IAT indicated the presence of either a weak or partial D phenotype. Genetic examination by RBC-READY GENE weak D test or DNA sequencing identified four types of weak D dubbed *RHD*15* (n=12), *RHD*01W.2* (n=3), *W.3*(n=1) and *W.9* (n=1).

The second group comprised DEL variants based on the negative IAT results and the positive results of the ads/el test. Genotyping identified 3 types of *RHD* alleles: *RHD*11* (n=5), *RHD*DEL8* and a variant with *RHD(IVS1-29G>C)* mutation in each sample.

The third group (n=7) comprised Dneg samples with three fragments of *RHD* and the negative ads/el result. In 2 cases, *RHD*DEL8* was detected with the commercial test and confirmed with DNA sequencing. In 5 cases, DNA sequencing revealed: *RHD*01N.23* and two novel alleles: *RHD*(767C>G)* and *RHD*(1029C>A)* in 3 and 1 case, respectively.

The newly described alleles (Fig. 2) have been submitted to the EMBL Nucleotide Sequence Database under accession numbers HE814563 and HE814564.

Unfortunately, whole blood from 8 donors was unavailable for further re-examination.

DISCUSSION

Prevention of alloimmunization by D antigen, especially in female of childbearing age, is the basic challenge for transfusion medicine. During the last decade, the knowledge of the genetic background of Dpos and Dneg phenotypes has opened new possibilities for improvement in accurate determination of the Rh phenotype.[11–14] Molecular strategies for Dneg variant identification that may be capable of immunization among donors typed as Dneg have been reported recently.[13–15, 28] Most published data is restricted to donors with C

or E antigens or with discrepant serological results.[16, 21–22] Presented *RHD* genotyping was focused on consecutive Polish donors routinely typed in RBTCs as Dneg. Our study is the first to present the molecular background of D negativity in the Polish population comprising a large number of donors that derive from very different regions of the country. The majority of our Dneg donors lack the whole *RHD* gene which is consistent with data published for other European countries.[2] In our cohort, we identified 63 of 31,200 apparently Dneg donors to harbor at least one fragment of the *RHD* gene tested by primers and probes specific for exons 7, 10 and intron 4. All *RHD*pos individuals were associated with C or E antigens. In 27 cases, the RhD protein was detected on the RBC surface. In 17 cases, weak D donors (type 2, 3, 9, 15) were mistyped as Dneg and in 10 cases only the ads/el test was sensitive enough to visualize the presence of D antigen. The remaining 20 donors out of 47 *RHD*pos / RhDneg donors available for serology were *RHD*pos but still D antigen negative.

The frequency of *RHD*pos donors was about 0.2%. Data published for Germany[28], Canada[17] or Austria[18] were between 0.2–1%. However, a comparison of data is not easy due to the differences in RhD groups tested and methods applied.

Firstly, the serological methods routinely used for D-negativity determination differ between countries.[16–19, 28–29] The Polish guidelines for D typing in blood donors recommend using two anti-D monoclonal reagents; at least one detects a weak expression of D antigen (including DVI). However, the methods used in various centers differed from one another. Large centers use automated systems based on microplate and/or microcolumn techniques or a system based on Capture technique. Small centers typed D antigen manually using a microcolumn test or a combination of tube NaCl and IAT.

Secondly, in our investigation we tested all Dneg donors independently from their RhCE status as there was no data collected from any Central-East European population. The molecular background in Poland might differ from other results performed in Europe.[16,18,22–24,28] We didn't "catch" any *RHD*pos donor with a C-c+E-e+ phenotype, although *RHD* screening for other populations revealed some cases among ccdee donors with a reduced D antigen expression on the surface of their RBC.[18,28]

And thirdly, the *RHD*-specific markers applied for *RHD* screening are crucial for identifying a Dpos donor. We chose a fragment of the intron 4 as the most suitable for gene screening since the exon and intron 4 are present in a majority of *RHD* variants with D expression.[3] Moreover, the region gives no false results in weak D type 4.3.[18] Similarly, the test in Germany was performed with the primers for that fragment due to the fact that its specificity for the *RHD* polymorphism in the intron 4 allows to search all clinically relevant *RHD*pos/ RhDpos variants, but misses the *RHD-CE-D* hybrids associated with the lack of D expression with the exception of Dpos hybrids, like *DVI*, that may be easily detected by standard serology.[28] Additionally, we examined a marker located in exon 7 because the molecular filter based on at least 2 fragments of the *RHD* gene makes the donor screening more reliable especially in the pooled material. However, due to the lack of discrepant results between these two tested regions (intron 4 and exon 7) it's no use to amplify both of them in the future screening.

For population study of our Dneg genetic background we also applied primers and a probe for exon 10 of *RHD* to discover any hybrid variants with long substitution of the *RHCE* considered not to express D antigen.[3,4]

In the presented work, a weak D type 15 encoded by 845G>A polymorphism was the most prevalent allele found among the seemingly Dneg Polish donors. The variant is described as partial weak D with extremely low density of D antigen but prone to alloimmunization and found mainly among Asians.[30] Occasionally it was described before in the European population but not with that high frequency of our study.[1, 4, 24] The type is also frequently observed in a group of donors with discrepant RhD results reported by our RBTCs (unpublished data). The variant expresses less than 200 antigens per cell[10], therefore, to a certain degree, labeling RBCs carrying type 15 as Dpos or Dneg depends on the accidental sensitivity of the applied methods routinely used in the RBTCs. Serologically it was recognized as *DFR* category. Similarly, an intermediate of *DFR* and *DVI* reaction pattern was reported by Wagner et.al for another partial *DIM* variant encoded by *RHD*(854G>A)* localized 2 amino-acids further.[31] The above observations indicate that SNP alterations in that part of exon 6 strongly effect RhD protein conformation not only by reducing the antigen density but also by changing the variant phenotype. In our study, the *RHD**15 is combined with the presence of E which is in agreement with observations of others.[1]

Our results demonstrate that weak D type 11 (11% of all *RHD*pos carriers) is a common variant in Polish D negative donors as described for other European countries.[1, 18, 23, 24] Even if the *RHD*11* phenotype was reported by Kormoczi et. al to exhibit the highest D antigen expression among RhD types determined as DEL, the *RHD* pool genotyping is the only way to prevent mistyping the *RHD*11* in routine serological testing.[10,11]

In our cohort, we identified 3 *RHD*DEL8* (*RHD(IVS3+1G>A)*) gene formations previously described by Wagner et al. and introduced into the DEL family.[2] Nevertheless, in our investigation we obtained two negative results in the ads/el test even after retesting the newly collected samples probably due to technical limitations. Kormoczi et al. and Polin et.al reported similar D negative serological results for the aberrant D variant.[10, 18] Its population frequency in Europeans was estimated about 1:15,152 and it approximates the calculations for the Polish population.[2] So far, no data are available on inducing allo-anti-D by the *RHD*DEL8* variant after transfusion to a D negative recipient but generally DEL phenotypes carry D molecules on the RBC surface. Thus, all such donors are transferred to D positive donor pool.

Among our seemingly Dneg donors we found 3 cases of *RHD*pos individuals without exon 10 detected but with D antigen present on the RBC's surface. A sequence analysis of cDNA confirmed the presence of a transcript containing only 9 exons. The exon 10 of the *RHD* gene was completely deleted and substrated with exon 10 of the *RHCE*. A comparable *RHD* allele was observed in Spanish[23], French[32] and Chinese[33] individuals with DEL phenotype. In our serological reinvestigation of all these samples, the D antigen was also visualized with the ads/el technique only. Other D variants with alteration in exon 10 of *RHD*, reported by Fichou et al. demonstrated the D antigen density about 30 molecules per RBC.[34] It might be implicated that a lack of the RhD cytoplasmic tail, encoded by exon 10, significantly reduces

the level of D protein below the limits of sensitivity for serological tests. What is interesting, Huang et al. demonstrated in the family study that such hybrid was combined with a D--haplotype.[33] Our findings indicate the presence of both *RHCE* alleles and exclude the same pathway of genomic rearrangements in the *RH* locus. This is an example of the dynamic nature of the *RHD* gene which leads to independent formation of the same aberrant allele. The population frequency of the mutation in Poland is comparable to *RHD*DEL8* variant described above and it should be taken into account in designing any *RHD* Polish donor screening. The study performed in Denmark with an initial test only for exon 10 might have missed that variant.[22] So it's worth emphasizing that the examination of exon 10 is not useful for the Dneg donor reevaluation program and should be omitted in the Polish population.

Among 47 *RHD*pos donors (available for the serological reevaluation using set of monoclonal anti-Ds for D categories and by ads/el technique) only in 20 cases (42.5%) RhD antigen was not present. Our screening detected exon 10 of the *RHD* to establish the spectrum of Polish *RHD-CE-D* alleles. The hybrid variants with a long substitution of the *RHCE* gene are quite common among Dneg donors in Europe. [2,4] In Poland, the *RHD-CE(2-9)-D* allele was the most frequent among our donors typed as D negative.

The present study identified two novel variations (c.767C>G, exon 5 and c.1029C>A, exon 7) in 3 and 1 RhDneg individuals, respectively (Fig. 2). These variants are expected to lead to an inactive *RHD* gene owing to premature in-frame stop codons [*RHD*(S256X) and *RHD*(Y343X)] and are the likely cause of the observed Dneg phenotype. This increases the total number of nonsense mutations responsible for Dneg phenotype to 14.[2,4,16,28,35_38] It's worth emphasizing that the *RHD*(767C>G)* allele associated with C was identified in 3 donors (5%) from different RBTCs in Poland with the population frequency comparable to the well-known variant *RHD*DEL8* synonymous *RHD(IVS3+1G>A*). This indicates that the new polymorphism is quite common in our country and may influence the results of *RHD* donor screening in the future.

One point is also worth underlying. 27/47 donors (all with C or E in their Rh haplotype) appeared to be actually weak D or DEL variants. Such donors are known to cause anti-D immunization post transfusion to Dneg recipient and therefore should be transferred to the Dpos donor group. The D status of 27 donors (0.09% out of 31,200) was changed. In an Austrian study about 0.3% of Dneg donors were reclassified as Dpos due to their real D phenotype revealed by molecular screening[18] and in a Danish report 1.7% of donors were established to be Dpos.[22] The *RHD* plasma-pool strategy has opened new path for verification of RhDneg results in a macro-donor scale which cuts costs dramatically. Some D variants with weak D or DEL expression may escape in routine typing.[39] The main advantage of *RHD* genotyping is an accurate identification of the aberrant allele within one test. If the *RHD* allele is described genetically and characterized by serology in local population, the decision of classifying a donor to Dpos or Dneg group may be based on the *RHD* genotype only. After adjusting *RHD* screening to the local polymorphisms in Polish Dneg donors, the plasma pooling assay will be determined routine in Poland. We are planning to abandon the exon 7 and 10 genotyping in favor of the intron 4 alone for plasma-pool screening. Prior to allele identification, it is necessary to design and optimize new

primers or probes complementary to novel SNP regions. A comprehensive algorithm for RhD verification of donor status is currently prepared (Fig.3). Taking into account our results, the *RHD* screening with intron 4 restricted only to RhDneg donors with C or E in their phenotype allows determining the *RHD* variant in 2% of such donors. According to our results the D antigen is present in 85% (29 out of 34) of such cases. The other *RHD*pos samples of RhDneg donors will require additional molecular characterization to exclude any false DEL results. Thus, from the economic point of view, it's worth to abandon further identification of the remaining 15% of *RHD*pos allele which are probably SNPs occurring with very low frequency. But due to the shortage of RhDneg RBC units such algorithm needs to be discussed. Additionally, the high prevalence of the *RHD* gene in the proposed scheme requires modification of the pool size.

In summary, the *RHD* gene was determined in 0.2% of Polish Dneg donors. In 50% of those samples, D antigen was detected. The study results indicate slight differences in the distribution of *RHD* alleles between the Polish population and other Caucasians.

The *RHD* genotyping strategy with DNA from plasma pools of Dneg carriers seems a useful method for mass screening of *RHD* variants. It helps to overcome the serological limitations in a simple and cost-effective manner, hence preventing immunization events according to the basic rule of D antigen compatible transfusion.

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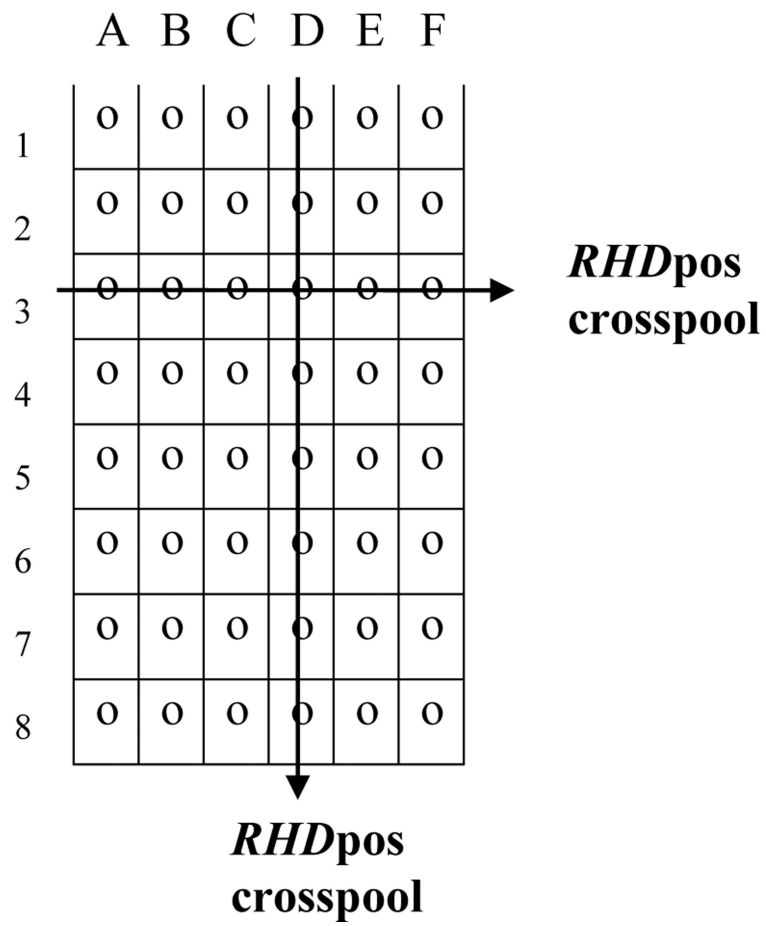

Figure 1.

The flow of cross-pooling strategy done in case of *RHD* gene detection in the 48-pool prepared from plasma. Samples from each column or row of the archive plate are combined to create 14 cross-pools. After DNA isolation *RHD* detection of 14 samples reveals two *RHD*pos cross-pools (plasma from a column #D and a row #3). Crossing numbers of *RHD*pos column and *RHD*pos row allows to identified an individual *RHD*pos sample on the archive plate (in D3 position).

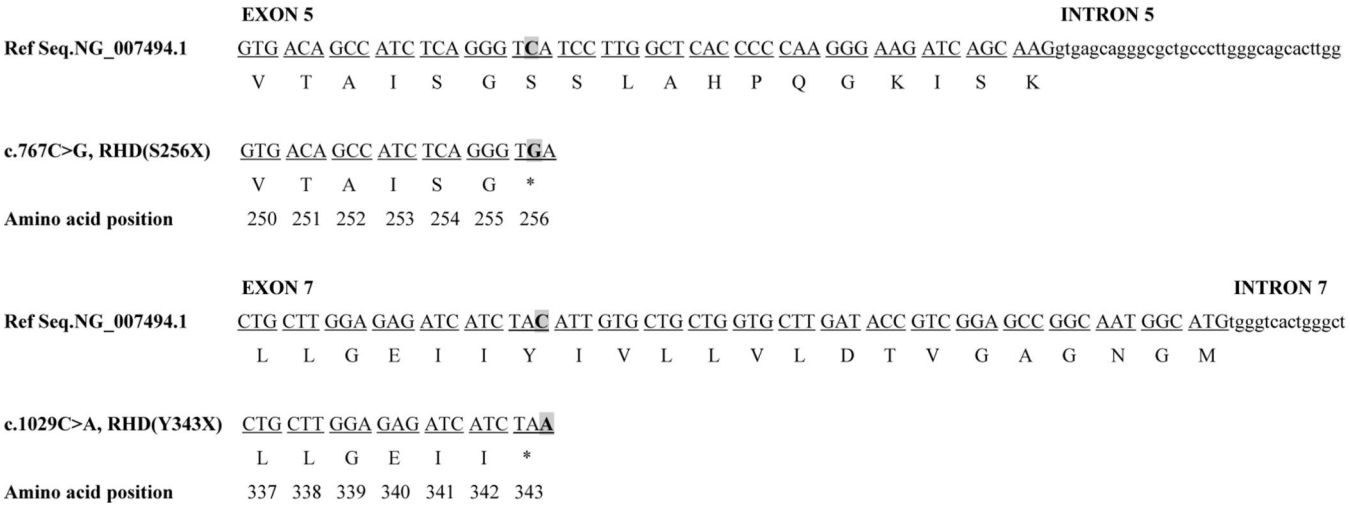

Figure 2.

Nucleotide sequences for *RHD*(S256X) and *RHD*(Y343X). Pertinent nucleotide and amino acid sequences for: 767C>G in exon 5 (NG_007494.1, position:29144..29230) and 1029C>A in exon 7 (NG_007494.1, position:34176..34255).

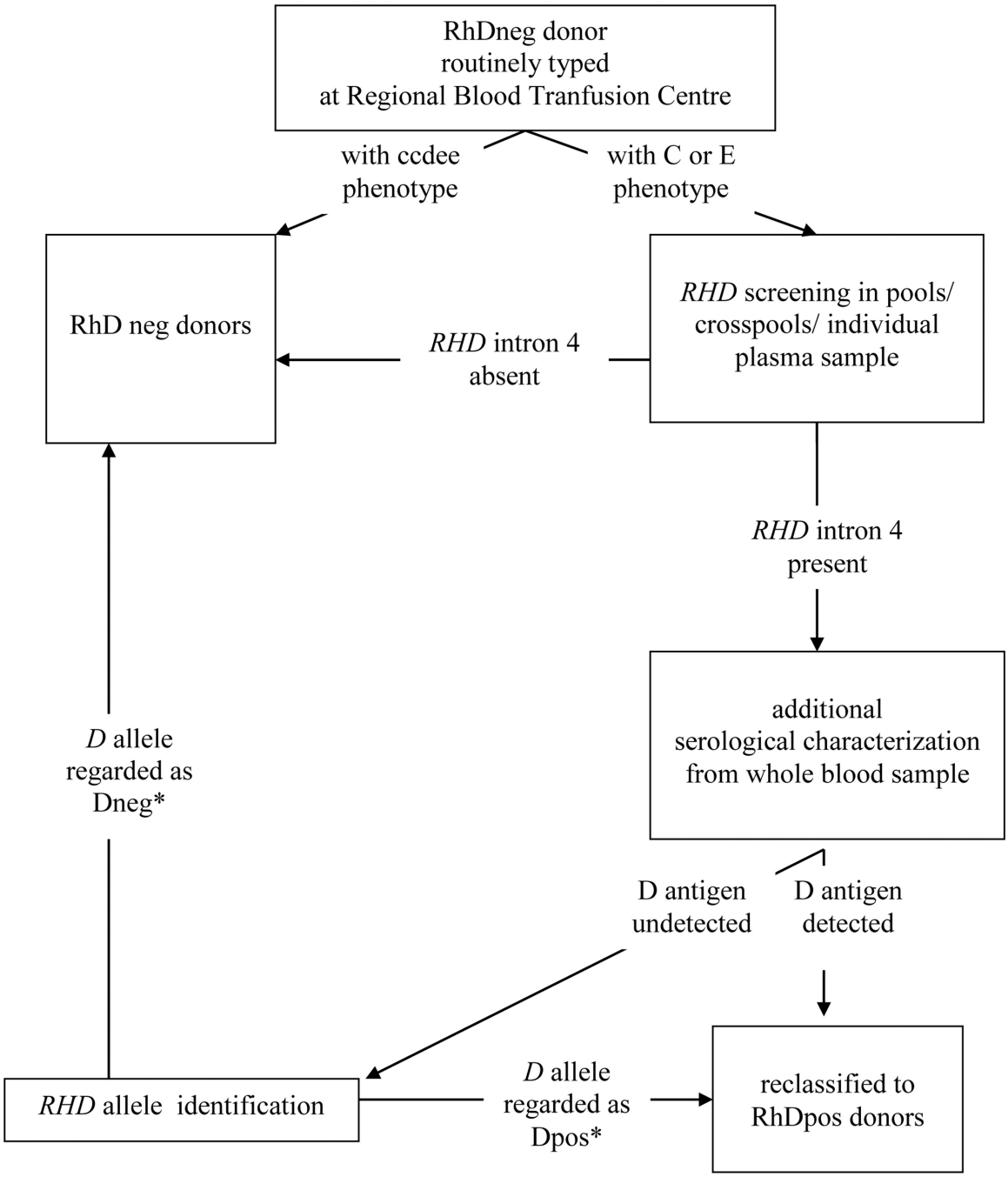

Figure 3.

An algorithm of classifying seemingly RhDneg donors to Dpos or Dneg group.

*according to Rhesus base/ EMBL

TABLE 1

Results of real-time PCR for *RHD*-specific markers located in intron 4, exon 7 and 10.

***RHD* results for:**			**Number of Dneg donors**
Intron 4	**Exon 7**	**Exon 10**	
−	−	−	31,137
−	−	+	19
+	+	−	3
+	+	+	41

TABLE 2

Characteristics of *RHD*pos donors routinely typed as RhDneg; estimated allele frequencies and 90% confidence intervals (CI) calculated according to the population's Poisson distribution.

Serological characteristics	Genetic characteristics	ISBT nomenclature	Population frequency	90%CI	Rh	No of samples
weak or partial D (n=17)	weak D typ 15	*RHD*15*	1:2,600	1,605–4,506	ccEe	12
	weak D typ 2	*RHD*01W.2*	1:10,400	4,024–38,156	Ccee	3
	weak D typ 3	*RHD*01W.3*	1:31,200	6,577–608,187	ccEe	1
	weak D typ 9	*RHD*01W.9*	1:31,200	6,577–608,187	ccEe	1
DEL (n=10)	weak D typ 11	*RHD*11*	1:4,457	2,373–9,497	Ccee	5
	RHD(IVS3+1G>A)	*RHD*DEL8*	1:10,400	4,024–38,156	Ccee	1
	RHD-CE(10)	-	1:10,400	4,024–38,156	Ccee	3
	RHD(IVS1-29G>C)	-	1:31,200	6,577–608,187	Ccee	1
RhD negative (n=20)	*RHD-CE(2-9)-D*	*RHD01N*03*	1:1,835	1,714–5,058	Ccee	11
	RHD-CE(4-7)-D	*RHD01N*07*	1:31,200	6,577–608,187	Ccee	1
	RHD-CE(2-7)-D	*RHD*01N.05*	1:31,200	6,577–608,187	Ccee	1
	RHD(IVS3+1G>A)	*RHD*DEL8*	1:10400	4,024–38,156	Ccee	2
	RHD(343delC)	*RHD*01N.23*	1:31200	6,577–608,187	Ccee	1
	RHD(767C>G)	Accession#: HE814563	1:10,400	4,024–38,156	Ccee	3
	RHD(1029C>A)	Accession#: HE814564	1:31,200	6,577–608,187	ccEe	1
lack of material (n=16)	*RHD-CE(2-9)-D*	*RHD*01N.03*	1:1,835	1,714–5,058	Ccee	6
	weak D typ 11	*RHD*11*	1:4,457	2,373–9,497	Ccee	2
	RHD intron4, exon7, exon10 detected	-	-	-	Ccee	8