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Review

Molecular *RHD* screening of RhD negative donors can replace standard serological testing for RhD negative donors

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ABSTRACT

This work aims to assess the value of a generalized molecular *RHD* screening strategy which could replace routine serological screening of weak D by indirect antiglobulin test. Three independent studies were performed at the two Blood Transfusion Services Berne and Zurich. Donors investigated were 652 RhD negative, but RhC and/or RhE positive, 17,391 mainly Rhccee, and 8200 with normal RhCcEe phenotype distribution. In study I single samples, in studies II and III minipools of 24 and 20 donor samples were tested, respectively. Among 26,243 phenotypically RhD negative blood donors, 65 carriers of *RHD* alleles were identified. Thirty-one of them were redefined as RhD positive.

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1. Introduction

The Rh blood group system is one of the most important and complex of the 33 current human blood group systems [1]. After ABO, it is the most clinically important blood group mainly due to its high immunogenicity [2,3]. The major antigens RhD, RhC, RhE, Rhc and Rhe, are encoded by two adjacent gene loci, the *RHD* gene which encodes the RhD protein with the RhD antigen and the *RHCE* gene which encodes the RhCE protein with the RhC, RhE, Rhc and Rhe antigens [4]. The close proximity of the two genes on chromosome 1, as well as, the arrangement of the *RHD* and *RHCE* genes at the *RH* locus promotes genetic exchange which in turn has created many clinically relevant Rh antigens [5]. Certain of these variants (e.g. DEL) are difficult to detect serologically and thus are often mistyped as RhD negative by standard serological assays [6]. Since some of these RhD variants have been documented to cause alloanti-D immunisations in RhD negative patients after transfusion [6–10], a comprehensive study of RhD negative donors is essential to identify red blood cell (RBC) units capable of inducing anti-D in RhD negative patients.

Several molecular studies in the Caucasian populations have been published during the last decade suggesting this approach will help identify the RhD variants, which with serological standard methods are difficult to detect [6,7,11–15]. Future molecular screening strategies among RhD negatives need to identify all the blood donors and their respective blood products which can potentially cause RhD-alloimmunisation. However they should not, if possible, increase the total cost of routine RhD antigen typing.

We aimed to gather molecular *RHD* data in three studies conducted on the Swiss RhD negative donor population. Of the in total 26,243 serological RhD negative donors investigated, after the molecular *RHD* screen, 31 donors were redefined as RhD positive. Most of them (28) had the phenotype RhCcee and none was Rhccee.

2. Material and methods

2.1. Donors

Study I included 652 consecutive RhD negative donors from four Swiss regional Blood Transfusion Services (BTS) with known positivity for RhC and/or RhE. Study II included, 17,391 consecutive RhD negative predominantly Rhccee donors from 12 of the 13 regional Swiss BTS with the exemption of Zurich. A small percentage (~3%) of these were RhC/E positive as not all regional Swiss BTS routinely phenotype their donors. In study III, 8200 RhD negative donors from BTS Zurich with a normal RhCcEe distribution were investigated.

2.2. Screening reagents

The RhD antigen status of blood donors was determined at the regional BTS with different methods; however two distinct anti-D IgM monoclonal antibodies and serological screening for weak D by indirect antiglobulin test (IAT) (first and second donation) were always employed, according to the Swiss Red Cross (SRC) BTS guidelines. All molecular *RHD* positive samples from study I and II were reanalysed at BTS Berne by ID/IAT (IgG card, BioRad, Cressier sur Morat, Switzerland) using a monoclonal anti-D (Clone ESD1, Bio-Rad), and at BTS Zürich, by IAT using anti-Dduo IgM/IgG, clone TH28/MS26 (Immucor, Roedermark, Germany).

2.3. Testing for DEL by adsorption/elution at BTS Zurich

Sample RBCs were washed three times in 0.9% NaCl (Diluent N.A., BioRad, Cressier sur Morat, Switzerland), mixed with Anti-Dduo IgM/IgG, clone TH28/MS26 (Immucor, Roedermark, Germany) at 1:3 (vol:vol) and incubated at 37 °C, 60 min. After 6 wash cycles using Diluent N.A., the RBC pellet was resuspended in cold (4 °C) 0.9% NaCl (Dr. G. Bichsel, Interlaken, Switzerland) at 1:1 (vol:vol). The suspension was then completed with cold (4 °C) Glycin/HCl buffer, 0.1 M, pH 3.0 at 1:1 (vol:vol) and incubated in ice water (<4 °C) for 60 s. After centrifugation (3000 U/min) for 2 min the recovered supernatant was neutralised with Na_2HPO_4/KH_2PO_4 buffer 0.8 M, pH 8.2 (Sigma–Aldrich, Buchs, Switzerland) at 10:1 (vol:vol) and assessed for the presence of Anti-D. If the eluate contained Anti-D, the phenotypic expression of *RHD* allele was proven.

2.4. Flow cytometric measurement of RhD density

The RhD antigen expression of some *RHD* positive samples were determined by flow cytometry according to previously described method [9,16] on a FACScan (Becton Dickinson, Heidelberg, Germany) with antibodies BS221, BS229 and H41 (provided by Biotest, Dreieich, Germany). As a secondary antibody, a goat anti-human IgG, Fab-fragment and FITC-conjugated was used (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). An RhD negative control sample was used to determine background fluorescence. A sample with 10,250 RhD antigens was used as a standard (provided by Günther Körmöczi, Vienna, Austria).

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2.5. Molecular biology

In study I, DNA was extracted from 200 µl EDTA blood from single donors using the QIAamp DNA Blood Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's instructions. In study II, blood samples were first pooled in batches of up to 24 samples on a Tecan pipetting robot (Tecan, Männedorf, Switzerland) before nucleic acid extraction using the QIAamp BioRobot EZ1 Blood kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's instructions. The DNA samples (study I and II) were tested for *RHD* specific polymorphisms in *RHD* exons 3, 5 and 10 by PCR using sequence specific priming (PCR-SSP) using primers as described previously (Table 1, [17]). Pools which tested positive in the PCR-SSP were resolved to the single donation by extracting each single blood sample and then retested using the same PCR-SSP. A pool containing 1:96 of *RHD* positive blood was used as a positive control. This pool showed an equal sensitivity to that of a single *RHD* positive blood sample. An *RHD* negative sample was used as negative control (data not shown).

In study I and II samples positive for at least one of these three *RHD* exons were further investigated with a commercial partial D type kit, targeting all *RHD* exons (Partial D type BAG Health CareGmbH, Lich, Germany, or RBC Ready Gene CDE (Innotrain GmbH, Kronberg, Germany). Nucleotide sequencing of the 10 *RHD* exons including adjacent flanking intronic regions was performed essentially as described in earlier publications [17,18]. Primers are given in Table 1. PCR was performed with a Qiagen HotStar Taq polymerase (Qiagen AG, Hombrechtikon, Switzerland). PCR products were purified and sequenced at the Microsynth DNA service facility with the dye terminator technology on an Applied BioSystems Genetic analyser (Microsynth AG, Balgach, Switzerland). The DNA sequences were analysed against *RHD* wild type sequence (Accession Number NG_007494.1) using the ClustalW2 multiple sequence alignment programme (Ref. http://www.ebi.ac.uk/Tools/clustalw2/index.html).

In study III, samples were screened in pools of 20 RhD negative donors, for *RHD* intron 4 and exon 7 only, as described previously [6]. Pools positive for intron 4 and/or exon 7 were resolved by analysis of all four preformed subpools of five donor's samples each, followed by the individual sample analysis of the positive subpool. DNA preparation of pools, subpools and single DNAs was done using the same method as described in study I. Pooling was done using a Tecan pipetting robotor (Tecan). Samples with genetic positivity for *RHD* in any of the specificities analysed, were further investigated by *RHD* exon scanning, detection of *RHCcEe*, testing for weak D types 1–5, 11, 15 and 17, *RHD* zygosity detection and testing for *RHD* del and *RHD* null alleles using commercially available PCR-SSP genotyping kits and according to the manufacturers' instructions (Inno-Train GmbH, Kronberg i. T., Germany). In cases where *RHD* allele present could not be classified by routine diagnostic PCR-SSP procedures, sequencing of the complete *RHD* gene was performed at the Institute for Clinical Transfusion Medicine and Immunogenetics in Ulm (Ulm, Germany), as described previously [19]. Only one sample, e.g. the newly described *RHD*IVS7-1G>A*, was sequenced in Linz, Austria as described previously (Red Cross Transfusion Center of Upper Austria) [13].

2.6. Classification criteria

The data bank from the University of Ulm (http://www.uni-ulm.de/~fwagner/RH/RB/) was chosen as reference for the *RHD* allele classification. All known mutations are declared according to the Ulm data bank as being either RhD positive or RhD negative. Samples with new *RHD* alleles or alleles where no mutation could be established, being RhD negative with standard serological testing, were analyzed using the adsorption/elution test. If the test was positive the donor was reclassified as RhD positive, whereas if the test was negative the donor remained RhD negative.

3. Results

3.1. Study I

Twenty-three out of 652 samples (3.53%) were PCR positive for at least one of three *RHD* exons analysed. Twenty-one of these had the phenotype RhCcee, two were RhCCee, whereas none had the RhccEe or RhccEE phenotype (Table 2). The alleles found were as follows: four *RHD*weak D type 11* (*RHD*M295I*), one *RHD*weak D type 31*, two *RHD*DVL-2*, three *RHD*IVS3+1G>A*, two *RHD*delEx10.5.4kb*, two *RHD*CE(2-9)-D*, three *RHD*CE(3-9)-D*, one *RHD*CE(4-9)-D*, two *RHD*DIIIa-CE(4-7)-D type 1* and three samples showing no specific mutation in all 10 *RHD* exons and flanking intron or promoter regions (Table 2). Blood donors with the following phenotypes; weak D type 11 (RhD(M295I)) weak D type 31, RhD(IVS3+1G>A),

Table 1
Primers used for the amplification of *RHD* exon 1-10 and for sequencing according to [17,30].

RHD exon	Forward primer	Reverse primer	Sequencing primer	Amplicon size (bp)
1	RHDpro-132F	RHD_i1+578R	SYpro-118F	903
2	nRHD_i1-1405F	RHD_i2+61R	SYi1-147F	1702
3	Ds3-s (rb20d)	Ds3-a	Ds3-seq (rb21) Ds3-s (rb20d)	219
4/5	nRHD_i3-45F	RHD_i5+149R	SYi4+103R SYi5+127R	982
6	nRHD_i5+1463F	nRHD_i6+57R	SYi5-149F	416
7	nRHD_i6+160F	nRHD_i7+326R	SYi6-130F	665
8	nRHD_i7+327F	nRHD_i8+151R	SYi7-121F	602
9	nRHD_i8+67F	nRHD_i9+62R	SYi9-58R	268
10	Ds10-s (re91)	Ds10-a (rr4)	Ds10-seq (rr3)	381

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Table 2
Summary of the *RHD* alleles found in studies I–III. Bold values stands for expressing RhD antigen.

RHD allele	Phenotype				I, BTS Berne (n = 652) C, E pos only	II, BTS Berne (n = 17,391) Mainly ccee	III, BTS Zurich (n = 8,200) All	Total (n = 26,243) C, c, E, e abnormal	RhD status
	RhC	Rhc	RhE	Rhe					
*RHD*Ψ*	–	c	–	e	–	3	4	7	neg
*RHD*Ψ*	C	c	–	e	–	1	–	1	neg
*RHD*delEx2*	–	c	–	e	–	1	–	1	neg
*RHD*CE(2-9)-D*	C	c	–	e	2	5	n.d.	7	neg
*RHD*CE(3-7)-D*	C	c	–	e	–	3	n.d.	3	neg
*RHD*CE(3-9)-D*	C	c	–	e	3	–	n.d.	3	neg
*RHD*CE(4-9)-D*	C	c	–	e	1	–	n.d.	1	neg
*RHD*CE(4-9)-D*	–	c	E	e	–	1	n.d.	1	neg
*RHD*DIIIa-CE(4-7)-D*	C	c	–	e	2		n.d.	2	neg
*RHD*DIIIa-CE(3-7)-D*	C	c	–	e	–	–	1+	1+	neg
*RHD*W16X*	C	c	–	e	–	–	1	1	neg
*RHD*343delC*	C	c	–	e	–	1	–	1	neg
*RHD*545delCTGT*	–	c	–	e	–	1	–	1	neg
*RHD*IVS7-1G>A*$	C	c	–	e	–	–	1	1	neg
RHD exon 1-10 WT			–	e	3	–	–	3	neg
*RHD*M295I* (*weak D type 11*)	C	c	–	e	**4**	–	**5**	**9**	**weak D(1) DEL(8)**
*RHD*IVS3+1G>A*	C	c	–	e	**2**	**1**	**4**	**7**	**DEL**
*RHD*IVS3+1G>A*	C	–	–	e	**1**	–	–	**1**	**DEL**
*RHD*K409K*	C	c	–	e	–	–	**1**	**1**	**DEL**
*RHD*IVS9-4064del5406*	C	c	–	e	**2**	–	–	**2**	**DEL**
*RHD*delEx10.5.4kb*	C	c	–	e	–	–	**1**	**1**	**weak D**
*RHD*DVL-2*	C	c	–	e	**2**	–	**4**	**6**	**weak D**
*RHD*IVS3+5G>A*	–	c	E	e	–	**1**	–	**1**	**weak D**
*RHD*weak D type 31*	C	–	–	e	**1**	–	–	**1**	**weak D**
*RHD*weak D type 38*	C	c	–	e	–	1	1	**2**	**weak D**
Total					**23**	**19**	**23**	**65**	

n.d. Large *RHD*CE-D* hybrid-alleles undetectable by the molecular method used. "+" marked sample is compound heterozygous for *RHD*DIIIa-CE(3-7)-D* and *RHD*Ψ*. "$" marked sample has a novel *RHD* allele with adsorption/elution negativity for RhD.

RhD(IVS5-38del4) and DVL2; were redefined as RhD positive due the potential of these variants to induce alloimmunisation. All other donors remained RhD negative.

3.2. Study II

Of the 17,391 serologically RhD negative donors, screened in minipools of up to 24 donations, were 19 donations positive with at least one of the three *RHD* exons (Table 2). Five of these donations were subsequently identified as having the phenotype Rhccee, which corresponds to 0.029% of all donations. Three were *RHD*Ψ*, one *RHD*delEx2* and one *RHD*545delCTGT* (Table 2). These five variants are all known to be phenotypically RhD negative [20–22].

Of the remaining 14 donors, were 12 RhCcee and two RhccEe. Among these 14 donors, one *RHD*weak D type 38*, five *RHD*CE(2-9)-D*, three *RHD*CE(3-7)-D*, one *RHD*Ψ*, one *RHD*343delC and one *RHD*IVS3+1G>A (Table 2) were identified. The RhccEe positive donations were found to be *RHD*CE(4-9)D and RHD*IVS3+5G>A* (Table 2). All these donor samples were provided by regional BTS's not routinely testing for the RhCE phenotype. Donors with the following alleles: *RHD*weak D type 38*, *RHD*IVS3+1G>A and RHD*IVS3+5G>A* were redefined as RhD positive because of their potential to induce alloimmunisation.

3.3. Study III

Of the 8200 donors screened in minipools of 20 donations at the BTS Zurich, were 23 molecularly *RHD* positive, which corresponds to 0.28% of a donor population with a normal RhCcEe phenotype distribution. Nineteen of 23 *RHD* positive samples showed the phenotype RhCcee, one due to expression of partial RhC from a *RHD*DIIIa-CE(3-7)-D* allele [23], present in compound heterozygosity with a *RHD*Ψ* in this individual. The remaining four were Rhccee and all *RHD*Ψ* carriers. The other *RHD* subtypes were: one RhD negative *RHD*W16X*, a novel *RHD*IVS7-1G>A*, with negative adsorption/elution for RhD, and five *RHD*M295I*, four *RHD*IVS3+1G>A*, one *RHD*K409K*, one *RHD*delEx10.5.4kb* [24], four *RHD*DVL-2* and one *RHD*weak D type 38*. In total, 16 of 23 *RHD* positives had to be redefined as RhD positive.

3.4. IAT with anti-D

Routine serological screening for RhD at the different regional BTS's was negative. However, at BTS Berne (study I and II), when rescreening the *RHD* positive samples by IAT using the clone ESD1, one of four weak D type 11, one of four DEL (*RHD*IVS3+1G>A*) and two of two DVL-2 were found to be serologically positive. At BTS Zurich (study III), rescreening of *RHD* positive samples by IAT using clone

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TH28/MS26, delivered one of four DEL (*RHD*IVS3+1G>A*) and one of four DVL-2 with RhD positive results.

3.5. RhD density

The RhD average antigen densities were 66, 93, 162 and 98 antigens per cell (Table 3) of weak D type 11, weak D type 31, weak D type 38 and RhD(IVS3+5G>A) respectively, using clone BS221, clone BS229 and Clone H41 of Anti-D.

4. Discussion

This study was carried out to assess the *RHD* allele frequency in serologically RhD negative blood donors in Switzerland. A further aim was to determine the practical feasibility of implementing molecular *RHD* typing to a routine everyday setting.

In Berne, the molecular *RHD* screening strategies targeted the three exons 3, 5 and 10 of the *RHD* gene. The *RHD* exon 5 was chosen primarily with aim to detect the *RHCE* variant RHCE*ceHAR, a variant occasionally found in Central European populations, which is not always detected by routine RhD serological screening but has been shown to be responsible for alloimmunisation [25].

In Zurich, as described previously, only *RHD* intron 4 and exon 7 were used as anchor points for the screening [6]. Screening strategies excluding *RHD* exon 10 do not detect large hybrid alleles, commonly recognized as RhD negative, which may erroneously ignore a recent Chinese observation of an *RHD*CE(4-9)-D* with adsorption/elution positivity for RhD [26]. Two samples carrying this hybrid allele were found among the samples tested in Berne (Table 2). However, these samples were not tested with adsorption/elution as the classification criteria (at this time) designated this allele with an RhD negative status.

Nevertheless, our strategies compare well with other studies targeting either one exon (e.g. exon 10) to those targeting multiple exons and flanking introns [6,7,12,14,15].

In the first study 23 samples among 652 RhC/E positive, but RhD negative donors carried *RHD* specific exon sequences, which correspond to 3.53% of the total RhD negative donors tested. This number is similar to those described in comparable studies conducted in Central Europe varying from 2.7% to 5.23% [7,12,15].

Many of the variants identified are still serologically RhD negative and remain regularly negative as donors. These include the *RHD*CE-D* hybrid alleles *RHD*CE-(2-9)D*, *RHD*CE-(3-9)D and RHD*DIIIa-CE*(4-7)-D. On the other hand, donors harboring alleles which are known to show weakened RhD phenotypes or DEL alleles, such as *RHD*M295I*, *RHD*weak D type 31*, *RHD*delEx10.5.4kb; RHD*IVS5-38del4*, *RHD*DVL-2* and *RHD*IVS3+1G>A*, were redefined as RhD positive among our blood donors.

Though the majority of the pooled samples in study II derived from Rhccee donors, a small – but significant number (estimated 500) – were RhC/E positive donors. From these pooled RhD negative, RhC/E positive samples, 14 donations were found to be positive for one or more *RHD* exons. Similar to study I, the majority (9) of these donations carried *RHD*CE-D* hybrid alleles, which are considered to be serologically RhD negative (http://www.uni-ulm.de/~fwagner/RH/RB/). Two further samples, carrying the alleles *RHD*Ψ* and *RHD*343delC* were also considered as RhD negative (http://www.uni-ulm.de/~fwagner/RH/RB/). The three donors harboring the alleles *RHD*weak D type 38*, *RHD*IVS3+5G>A* and *RHD*IVS3+1G>A* were redefined as RhD positive (http://www.uni-ulm.de/~fwagner/RH/RB/).

Very few Rhccee donors were found to be positive for *RHD* exon sequences. Only 5 of 17,391 in Berne, which corresponds to 0.029%, and 5 of 8200 in Zurich, which were *RHD*Ψ* positives and corresponds to 0.061%. In a study performed in Austria it was found that 0.15% of the Rhccee samples were positive for the *RHD* gene [14]. Surprisingly they found 31 *RHD*DAR5* positive samples within their screen of 23,330 RhD negative donors [14]. Twenty-nine of these 31 samples were found in Rhccee positive donors and had escaped the routine serological RhD blood group typing. If donors harbouring the *RHD*DAR5* allele are excluded, the frequency is reduced to 0.014%, a figure similar to that detected in the present study. In any case, no donors with the Rhccee phenotype of our studies had to be redefined as RhD positive.

Although the majority of the known aberrant *RHD* alleles are associated with the antigens RhC or RhE, disregarding the donors with an Rhccee phenotype seems risky, since several weak D, DEL and RoHAR variants are associated with this phenotype [12]. For our needs in Switzerland, a decision was made to continue screening of all RhD negative individuals, independent of their RhCE phenotype, especially since approximately half of all RhD negative blood donors had already been included in studies I, II and III. On the other hand, costs are an ever present topic and serotyping RhD negatives with a potent anti-CDE, followed by a molecular *RHD* testing of the resulting positives, may represent a cost-efficient and productive alternative.

Table 3
RhD antigen densities of weak D type 11, 31, 38 and RhD(IVS3+5G>A). The samples were analysed by flow cytometry using three monoclonal antibodies (BS221, BS229 and H 41).

RhD variant	MFI*/antigen density			Mean antigen density per RBC
	BS221	BS229	H41	
Standard Vienna	320/10,250	180/10,250	180/10,250	10,250
Weak D type 11	1.75/56	1.54/88	1.70/54	66
Weak D type 31	2.31/74	2.33/132	2.31/74	93
Weak D type 38	4.03/129	3.89/221	4.22/135	162
RHD(IVS3+5G>A)	2.53/81	2.29/129	2.59/83	98

* MFI, median fluorescence intensity.

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The four variants, weak D type 11, 31, 38 and DEL (*RHD*IVS3+5G>A*), known to have very weak RhD phenotypes, where analysed by flow cytometry (Table 3), showing considerable weakened but detectable antigen expression. The RhD antigen densities of 66–162 (Weak D type 11, 31 and 38) were in accordance with other published data [9,27–29].

When repeating the serological testing (IAT using clone ESD1) of the 12 *RHD* positive donors in study I, it was observed that if this clone had been used, four of these donors (one *RHD*M29I*, one *RHD*IVS3+1G>A* and two RHD**DVL-2*) could have been found as serologically RhD positive. Similarly, in study III, one of four DEL (*RHD*IVS3+1G>A*) and one of four DVL-2 could have been identified as RhD positives by IAT using clone TH28/MS26.

The three major objectives of this study were to develop a *RHD* screening method, to determine the *RHD* allele frequency among RhD negative blood donors and to determine the number of donors that had to be redefined as RhD positive. It is clear that this approach would reduce the potential risk of immunization of patients as we redefined 31 previously RhD negatives to RhD positives of 65 *RHD* positives among 26,243 previously RhD negative donors. Studies II and III showed that pooling of up to 24 samples is practicable and suitable for a routine *RHD* screening setup. In addition, the accumulated data of that study (studies I–III) helped in the decision to introduce a mandatory molecular *RHD* screening program for serologically RhD negative blood donations in Switzerland.

Besides the development of *RHD* screening strategies these studies determined the prevalence of *RHD* gene carriers within the serologically RhD negative Swiss donor population and were used as a support in the decision making for the introduction of an mandatory molecular *RHD* screening program for serologically RhD negative blood donation in Switzerland.

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