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Review

Implementation of a mandatory donor *RHD* screening in Switzerland

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ABSTRACT

Starting in 2013, blood donors must be tested at least using: (1) one monoclonal anti-D and one anti-CDE (alternatively full RhCcEe phenotyping), and (2) all RhD negative donors must be tested for *RHD* exons 5 and 10 plus one further exonic, or intronic *RHD* specificity, according to the guidelines of the Blood Transfusion Service of the Swiss Red Cross (BTS SRC). In 2012 an adequate stock of *RHD* screened donors was built. Of all 25,370 RhD negative Swiss donors tested in 2012, 20,015 tested at BTS Berne and 5355 at BTS Zürich, showed 120 (0.47%) *RHD* positivity. Thirty-seven (0.15%) had to be redefined as RhD positive. Routine molecular *RHD* screening is reliable, rapid and cost-effective and provides safer RBC units in Switzerland.

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1. Introduction

There have been substantial discussions about whether or not *RHD* genotyping should be implemented as a routine assay for blood donors [1–5]. Based on published data [5], and data collected in previous studies (see Gowland et al., in this issue), the Blood Transfusion Service Swiss Red Cross (BTS SRC) decided that molecular *RHD* screening for all serological RhD negative donors should be mandatory from January 2013. In order to establish a stock of RhD negative donors screened for the *RHD* gene, 2012 was assigned as a transition year. This was done in order to avoid shortage of RhD negative red blood cell (RBC) units in 2013.

The general algorithm and the testing procedure are described in the national guidelines of BTS SRC (<https://sbsc-bsd.ch/DokuMan/DokumenteBSD/Allgemeines/tabid/107/language/de-CH/Default.aspx>). In brief, all donors, repeat, lapsed and first time donors having a negative serological RhD status have to be molecularly screened at least once for the *RHD* gene in exon 5 and 10 and one additional exonic or intronic *RHD*-specificity. As of January 2013 the molecular *RHD* screening is required for the release of RhD negative RBC units in Switzerland.

In order to obtain a more appropriate routine *RHD* screening system the previously used systems were optimized (Gowland et al., in this issue). The aim was to be more time and cost efficient with equal sensitivity and specificity. To achieve this, different measurements were considered. At the BTS Berne, pooling of up to 23 samples was kept. The assay was adapted to a single multiplex assay for *RHD* exons 3, 5 and 10 using a simplified nucleic acid extraction. Amplified products were analyzed by a capillary gel electrophoresis method with automatic read-out and transfer to the blood bank data system, instead of using three singleplex and agarose gel electrophoresis without automated gel reading. BTS Zürich, however, changed from pooled testing (Gowland et al., in this issue) to individual DNA extraction in an automated 96-well format, in order to avoid time consuming and costly pool resolutions. For molecular testing, *RHD* exon 5 and 10, and *RHD* exon 7 and *ABO^B* were multiplexed into two reactions and analyzed on agarose gels, loaded with multi-channel pipettes. An input mask for positive amplifications allowed further automated data-processing, whereas comparison of *ABO^B* positivity with the existing serological values excluded serial sample mix-ups.

As an additional action the testing for weak D by indirect antiglobulin test (IAT) was discontinued from the beginning of 2013 and thus no longer mandatory,

rendering the molecular *RHD* screening cost neutral. In summary, resources are minimized and the safety of RBC units has increased. Results from the first year of *RHD* screening show that the decision taken has already contributed to safer blood products. In 2012, thirty-seven donors from all over Switzerland were reclassified as RhD positive.

2. Material and methods

2.1. Procedure BTS Berne

2.1.1. Blood samples

EDTA blood samples from first time and non-tested consecutive RhD negative donors were pooled in pools of up to 23 donors using a Tecan Genesis liquid handling platform (Tecan, Männedorf, Switzerland). At the same time repository 96-well plates with EDTA whole blood of single donations were prepared for possible pool resolutions. Samples known to be positive for RhC or RhE were either pooled together or tested separately.

2.1.2. DNA extraction

Genomic DNA was extracted from whole blood using the REExtract-N-Amp™ Blood PCR Kit (Sigma, Saint Louis Missouri, USA) as described by the manufacturer.

2.1.3. Amplification

Amplification of the *RHD* gene was done by multiplex sequence specific primer (SSP)-PCR using three primer pairs targeting *RHD* exons 3, 5 and 10 respectively (Table 1). The multiplex also contained primers targeting the human growth hormone gene (HGH), used as a DNA extraction and amplification control (Table 1). PCR conditions in brief: 2 µl extracted DNA, primers in concentrations as listed in Table 1, 30 mM tetramethylammonium chloride solution (TMAC) (Sigma, Buchs, Switzerland), deionized water and 10 µl REExtract-N-Amp Blood PCR ReadyMix (Sigma, Buchs, Switzerland) were mixed in a final volume of 20 µl.

The amplification was performed on a T 3000 thermocycler (Biometra, Göttingen, Germany) using a program consisting of an initial denaturation at 94 °C for 6 min followed by 5 two-temperature cycles of 20 s at 94 °C and 60 s at 70 °C followed by 10 two-temperature cycles of 20 s at 94 °C and 60 s at 65 °C, followed by a 35 three-temperature cycles of 20 s at 94 °C, 30 s at 61 °C, and 1 min at 72 °C. The final elongation step of 15 min at

Table 1

Primers used for PCR-SSP multiplex-1 in Berne and multiplexes 1 and 2 in Zurich, respectively.

Multi-plex reaction	Location	Primer name	Primer sequence (5' > 3')	Final conc. (nM)	Length PCR-product (bp)
Berne-1	<i>RHD</i> intron 2	RHD_i2-188F	CAGAGGCCACCTTAACGGGA	250	275
Berne-1	<i>RHD</i> exon 3	D-3-380R-m1	GACCTTCCCCAAGACAGCATACA	250	
Berne-1	<i>RHD</i> exon 5	D-5-for	CAGGAGTGTGATTCTGGCCAACC	175	216
Berne-1	<i>RHD</i> exon 5	D-5-787-m1	CTGCTCACCTTGCTGATCTTACC	175	
Berne-1	<i>RHD</i> exon 10	D-10-for	GTTTCCTCATTTGGCTGTTGGATTTAAAGCA	400	149
Berne-1	<i>RHD</i> 3' UTR	D-10-1358long-m1	CCAGTGCCTGCGCGAACAGTG	400	
Berne-1	Control	HGH-for	TGCCTTCCCAACCATTCCTTATCC	100	434
Berne-1	Control	HGH-rev	CCACTCACGGATTCTGTTGTGTTCC	100	
Zurich-1	<i>RHD</i> exon 5	rdRH-ex5+676-F	GTTCTGGCCAAGTTCAACCCCTG	500	153
Zurich-1	<i>RHD</i> exon 5	rdRHD-ex5+787-R	CTCACCTTGTGATCTTCCC	500	
Zurich-1	<i>RHD</i> exon 10	RH-i9-69-F	CTAAGGTCTTGTAAATGAGACATTTAGGCTGTT	150	251
Zurich-1	<i>RHD</i> 3' UTR	RHD-ex10+1358-R	CAGTGCCTGCGCGAACATTG	150	
Zurich-2	<i>RHD</i> exon 7	RH-ex7+967-F	GTTGTAACCGAGTCTGGGGATTC	300	123
Zurich-2	<i>RHD</i> exon 7	RHD-ex7+1048-R	TGCCGGCTCCGACGGTATC	300	
Zurich-2	<i>ABO</i> *B exon 7	ABO-all+649-F	AGTGACGTGGACATGGAGTTCC	300	194
Zurich-2	<i>ABO</i> *B exon 7	ndABO-B+803-R	CCGACCCCCGAAGACCC	300	
1&2	Control	GH1+96-F	TGCCTTCCCAACCATTCCTTATCC	70	434
1&2	Control	GH1+274-R	CCACTCACGGATTCTGTTGTGTTCC	70	

72 °C was followed by a one-temperature step of 4 °C. A pool containing 1:95 of *RHD* positive blood was used as a positive control. This pool showed an equal sensitivity to that of a single blood sample. An *RHD* negative sample was used as negative control.

2.1.4. Post-PCR analyses

After amplification pools were analyzed by capillary gel electrophoresis on a QIAxcel system, using a QIAxcel DNA Screening Kit (2400) (QIAGEN, Hombrechtikon, Switzerland). This was performed according to the manufacturer.

The QIAxcel electropherograms were thereafter analyzed using the BioCalculator software (QIAGEN) and converted in to binary code (BIN-file).

2.1.5. Pool resolution into single samples

Pools positive for one, two or all three *RHD* exons were resolved into single donations using the repository plate and analyzed as described above.

2.1.6. Electronic data handling and transfer

Binary pool and sample data from the QIAxcel BioCalculator software were processed by the PCR_Tools_BSD Bern (Blood Transfusion Service SRC Berne, Berne, Switzerland) software, providing the necessary pool management and database functions for an automated process. Negative samples were released by PCR_Tools_BSD Bern and sent electronically by e-mail and fax to other blood transfusion services or exported to the AnaRes.NET software (EM-DAT, Bern, Switzerland), according their preferences. AnaRes.NET is a laboratory result management software solution (EM-DAT, Bern, Switzerland) working as a gateway between PCR_Tools_BSD Bern and the blood donor data bank, eProgesa (eProgesa, MAK-System, Paris, France).

2.1.7. Serologic characterization

Donor samples positive for the *RHD* exon 3, 5 and/or 10 were phenotyped for RhD with the polyclonal ID ABO/DD card and the polyclonal and monoclonal ID phenotype

cards (Bio-Rad). Testing for weak D by IAT was also conducted using a monoclonal anti-D (Clone ESD1, Bio-Rad, Cressier, Switzerland).

2.1.8. *RHD* allele characterization

Depending on the result of the serological characterization samples positive for *RHD* exon 3, 5 and/or 10 were further tested using either the BAGene Partial-D-TYPE kit or the BAGene Weak D-TYPE kit (BAG Healthcare GmbH, Lich Germany). In case of an ambiguous result samples were further tested by exon sequencing as described previously [6,7].

2.1.9. Adsorption/elution

For the adsorption/elution test two different anti-D were used, a monoclonal anti-D clone ESD1 (Bio-Rad, Cressier, Switzerland) and an anti-D pool (a pool of 24 immunized donors, 8 µg RhD/ml, in-house, BTS Berne). For the anti-D adsorption to RBCs, 250 µl of washed donor cells were incubated for 45 min at 37 °C with 250 µl of anti-D ESD1 or 100 µl of anti-D pool. The cells were washed and eluted by acid elution technique as described by manufacturer (Elution Kit, Almedica AG, Giffers, Switzerland). The eluate and supernatant from the last wash were used for IAT against RhD positive (RhCCDee and RhccDEE) and RhD negative (Rhccdde) cells treated with papain. The tests were carried out in ID/IAT cards (Bio-Rad, Cressier, Switzerland) applying 50 µl of 0.8% RBCs in modified LISS (Diluent II, Bio-Rad, Cressier, Switzerland) and 25 µl of eluate (or last supernatant).

2.2. Procedure BTS Zürich

2.2.1. Blood samples

EDTA blood samples from first time and non-tested consecutive RhD negative donors were tested. DNA was extracted from single samples in 96-well microtiter plates applying the Chemagen magnetic bead technology (Perkin Elmer, Baesweiler, Germany), using 200 µl EDTA blood

resulting in approximately 8 µg total genomic DNA (100 µl elute) and as described previously [8].

Serologic characterization (including adsorption/elution testing) was done as described in this edition (Gowland et al., this issue).

2.2.2. Amplification and post-PCR

Screening of single RhD negative DNAs was done in two multiplexed PCR-SSPs (MPX-SSP) detecting *RHD* specific nucleotides of exon 5 and 10 in MPX-SSP1 (Table 1) and exon 7 plus specificity for *ABO*B* in MPX-SSP2 (Table 1). Primers specific for human growth hormone (*GH1*) were added as positive amplification control to both reactions. Final MgCl₂ concentration was 1.5 mM in both reactions, all other PCR set up conditions were as described earlier [9]. Amplifications were carried out on either GeneAmp® PCR System 9700, or Veriti® 96-well plate cyclers (Life Technologies Europe B. V., Zug, Switzerland) and started with an initial denaturation step of 120 s at 94°, 5 incubation cycles for 20 s at 94°, 60 s at 65° and 45 s at 72°, 20 incubation cycles for 20 s at 94°, 50 s at 61° and 45 s at 72°, to finally end with an amplification at 72° for 300 s. Amplification products were loaded onto a 2% Agarose gel and UV documented. Identification of *RHD* alleles present was done as described previously (part A, Gowland et al., this issue). Sequencing of *RHD* alleles was performed in Linz, Austria (Red Cross Transfusion Center of Upper Austria) [10].

2.2.3. Classification criteria

Classification criteria were applied as described previously (Gowland et al., this issue, in this issue).

3. Results

In total, 25,370 serological RhD negative donors were screened molecularly for the *RHD* gene, in Berne (20,015) and Zürich (5355), respectively during the transition year 2012. Included in these data are all RhD negative donors from all 13 BTS in Switzerland observed in this time period.

In Berne a total of 982 pools were screened (in average 19.42 donations per pool), with 876 being truly *RHD* negative. Of the *RHD* positive pools, 74 were positive for all three *RHD* exons (3, 5, 10), five were positive for exons 3 and 10 and 27 for exon 10 only. Furthermore, six pools were positive due to weak D type 1, 2, 3, or 4. These donor samples were possibly added to the pools by mistake. In addition, 947 samples (RhC or RhE positive) were tested as individual samples, with 931 being truly *RHD* negative. Out of the *RHD* positive samples, nine were positive for all three *RHD* exons (3, 5, 10), one was positive for exons 3 and 10 and six for exon 10 only (Table 2).

In Zurich, 16 individuals of 5355 totally investigated RhD negatives showed *RHD* positivity. Eight donors were positive for *RHD* exons 5, 7 and 10, and eight showed positivity for exon 10 only (Table 2).

Of the 25,370 donors investigated in total, 120 (0.47%) were molecularly positive for the *RHD* gene (Table 2).

Thirty-seven (0.15%) of them were reclassified as RhD positive.

Four new *RHD* alleles were detected during the transition year 2012. The newly detected variants were deposited in the EMBL-EBI European Nucleotide Archive (www.ebi.ac.uk/ena/home). These included: mutation 872C>G leading to P291R in RhD, accession number HE999545; a mutation which has until now only been found in conjunction with T201R and F223V in weak D type 4.3 variants; mutation 46T>C leading to W16R, accession number HE999546; a deletion of G882 leading to *RHD**882delG, accession number HG779212 and mutation 335G>C, a splice site mutation at the last nucleotide of exon 2, leading to S112T, accession number HE999547. Red cells with W16R reacted weakly positive in the IAT while variants P291R, *RHD**882delG and S112T could only be detected by the adsorption/elution test. Thus, all these variants can be classified as DELs.

4. Discussion

After various reports of weak D and DEL variants that may cause alloimmunisation in RhD negative recipients [6,11–14], the concern of not being able to identify donors with weak D and DEL phenotypes by standard serological donor testing has increased. There are indications that sometimes very few RhD antigens are needed for immunogenicity as some DEL variants have proven to cause alloimmunisation [13,14]. It was originally believed that at least 300 to 400 RhD antigen sites per RBC would be required for immunogenicity, but that seems no longer to be true, as RBCs with fewer than 30 antigen sites have been shown to be immunogenic [6,11,15,16].

Therefore, and based on data of our own study results obtained from Swiss blood donors (presented in this edition), the BTS SRC set up a working group to decide whether the current serological RhD screening strategy should be adapted to include *RHD* genotyping, and if so, which strategy would be the most cost effective. The working group recommended the introduction of molecular *RHD* screening to complement the existing serologic RhD typing with the aim to reduce the occurrence of transfusion incidents. Based on this recommendation, BTS SRC decided to introduce a mandatory molecular *RHD* screening for all serological RhD negative (new and repeat) donors to begin in January 2013, with 2012 as a transition year.

Our strategy compares well with other studies having targeted only one (e.g. exon 10) to multiple exons including flanking regions [6,17–21]. In total 120 of 25,370 (0.47%) samples, serologically tested as RhD negative, were positive for the *RHD* gene. This number corresponds well with data from other countries such as Denmark, Austria and Germany, with 0.21% up to 0.59% of molecularly *RHD* positive donors in a serological RhD negative donor population [4,6,17–19]. In the course of our study, thirty-seven of the 120 (30.8%) *RHD* positive samples were re-declared as being serologically RhD positive. These data also correspond quite well with data from European countries, ranging from 15% to 49% [4,6,17,18]; In contrast, the study performed in Upper-Austria redefined as many as 79% of the samples being positive for the *RHD* gene [19]. However,

Table 2Summary of *RHD* alleles found among 25,370 RhD negative donors, 20,015 tested at BTS Berne, and 5,355 at BTS Zürich.

RHD allele	RhC	Rhc	RhE	Rhe	BTS Berne	BTS Zürich	IAT with anti-D (ESD1/TH28/MS26)	Adsorption/elution	RhD status
<i>RHD</i> *ψ	–	c	–	e	17	–	neg	n.a.	neg
<i>RHD</i> *545delCTGT	–	c	–	e	2	–	neg	n.a.	neg
<i>RHD</i> *343delC	C	c	–	e	1	–	neg	neg	neg
<i>RHD</i> *489delAGAC	C	c	–	e	1	–	neg	n.a.	neg
<i>RHD</i> *R318X	C	c	–	e	1	–	neg	n.a.	neg
<i>RHD</i> *W16X	C	c	–	e	4	1	neg/neg	n.a.	neg
<i>RHD</i> *CE(2-7)-D	C	c	–	e	2	–	neg	n.a.	neg
<i>RHD</i> *CE(2-8)-D	C	c	–	e	2	–	neg	n.a.	neg
<i>RHD</i> *CE(2-9)-D	C	c	–	e	26	1	neg/neg	n.a.	neg
<i>RHD</i> *CE(2-9)-D	C	–	–	e	–	1	neg	n.a.	neg
<i>RHD</i> *CE(3-7)-D	C	c	–	e	10	–	neg	n.a.	neg
<i>RHD</i> *CE(3-7/3-8)-D	C	c	–	e	–	1	neg	neg	neg
<i>RHD</i> *CE(3-7/3-8)-D	C ^w	c	–	e	–	1	neg	n.a.	neg
<i>RHD</i> *CE(3-8)-D	C	c	–	e	1	–	neg	n.a.	neg
<i>RHD</i> *CE(3-9)-D	C	c	–	e	3	–	neg	n.a.	neg
<i>RHD</i> *DIIIa-CE(3-7)-D	C	c	–	e	–	2	neg	n.a.	neg
<i>RHD</i> *CE(4-7)-D	C	c	–	e	–	1	neg	neg	neg
<i>RHD</i> *CE(4-9)-D****	–	c	E	e	2	–	neg	n.a.	neg
<i>RHD</i> *CE(4-7/4-8)-D	–	c	E	e	–	1	n.a.	neg	neg
<i>RHD</i> *IVS3+1G>A	C	c	–	e	6	1	neg/neg	pos	DEL
<i>RHD</i> *93insT	C	c	–	e	1	–	neg	pos	DEL
<i>RHD</i> *K409K	C	c	–	e	2	–	neg	n.a.	DEL
<i>RHD</i> *M295I	C	c	–	e	10	2	pos*/neg	n.a.	DEL
<i>RHD</i> *M295T	C	c	–	e	3	–	neg	pos	DEL
<i>RHD</i> *P291R***	C	c	–	e	1	–	neg	pos	DEL
<i>RHD</i> *S112T***	C	c	–	e	1	–	neg	pos	DEL
<i>RHD</i> *882delG***	C	c	–	e	1	–	neg	pos	DEL
<i>RHD</i> *delEx10.5.4kb	C	c	–	e	–	2	neg	pos	DEL
<i>RHD</i> *W16R***	–	c	E	e	1	–	pos*	n.a.	DEL
<i>RHD</i> *IVS3+5G>A	–	c	E	e	1	–	neg	pos	DEL
<i>RHD</i> *weak D Typ 31	C	c	–	e	2	1	pos*/pos*	pos	weak D
<i>RHD</i> *weak D Typ 38	C	c	–	e	1	–	pos*	n.a.	weak D
<i>RHD</i> *DVL-2	C	c	–	e	–	1	neg	n.a.	weak D
<i>RHD</i> unknown	C	c	–	e	1	–	neg	n.a.**	?
<i>RHD</i> exon 1–10 WT	C	c	–	e	1	–	neg	n.a.**	?
Total					104	16			

n.a.: adsorption/elution was not performed, as the serological status of these variants is described in the RhesusBase (<http://www.uni-ulm.de/~fwagner/RH/RB/>).

* Weakly positive.

** Adsorption has not yet been performed.

*** Newly detected variants.

**** *RHD**CE(4-9)-D previously also described as adsorption/elution RhD positive in one Chinese individual of RhCcee phenotype [22].

they found thirty-one weak D type 4.3 positive samples within their screen of 23,330 RhD negative donors [19]. Most of these samples were Rhccee and had escaped routine serological anti-D screen (inclusive retrospective testing by IAT). If donors carrying the *RHD**weak D type 4.3 genotype are excluded, the frequency of the reclassification would be reduced to be 45.7%, a figure similar to that detected in the present study.

We could show the practical and economic feasibility of the implemented molecular routine *RHD* screen, targeting *RHD* exon 5 and 10 plus either exon 3, or exon 7. In order to achieve an even better cost efficiency, the three target exons were multiplexed into one PCR reaction in Berne. Both automated data transfer systems to the in house IT databases, as well as to the regional BTSs have been validated thoroughly, showing to be robust and reliable. In the meantime, this way, more than 50,000 donor results have been transferred to our two blood bank systems or

electronically sent to other regional blood transfusion services. Automation, multiplexing and development of IT based reporting have extensively improved the molecular *RHD* screening. A well-defined, electronic and automated process leads, on one hand to an overall cost reduction and on the other hand to an increased safety of data transfer since the manual manipulation of data and thus human mistakes are reduced.

The cost for the molecular *RHD* screen is less than € 7.00 and less than € 10.00 per sample investigated in Berne and Zurich, respectively. These costs include sample handling (pipetting, not including the phlebotomy sampling tubes), DNA extraction (including pool resolution at BTS Berne), PCR-SSP multiplex typing, documentation & reporting, allele-identification of *RHD* positives (including sequencing, when necessary), and hardware (amortisation calculated within 3 years and the given sample numbers, and service-costs). Above mentioned costs include approximately

2 min. and 4 min. of hands on time per sample in Berne and Zurich respectively, which roughly corresponds to € 1.63 and € 3.25 personal costs in high priced Switzerland. Difference in costs between the two centres is dependent on the methods used. Whereas pooling and automated PCR-product readout saves costs in Berne, classical agarose gel electrophoresis and individual DNA extraction in Zurich are costly. However, in Zurich, individual DNAs are “recycled” in a search for blood donors with high-frequency antigen negativity, such as Kp(a+b-), Lu(a+b-), Yt(a-b+), Co(a-b+), and other genotypes, noteworthy, all of them negative for RhD [8].

Evidently, costs per donation would increase if the number of samples per pool were to decrease. In contrast, reduction of costs is provided by abolishing routine IAT on all serological RhD negative donors, which was mandatory according to the previous guidelines to capture weak D phenotypes. The cost of this previous RhD screening procedure outweighs the actual costs of molecular *RHD* screening. Here we propose a molecular approach representing a rapid, cost efficient strategy to obtain safer RBC units in order to prevent unnecessary RhD alloimmunisation. A zero risk is not a realistic goal for the routine molecular *RHD* screening, but compared to the situation with exclusive IAT assessment of RhD negativity the safety of the RBC units has now increased in Switzerland. Furthermore, the release of molecularly confirmed RhD negative RBC units in between 72 h after donation guarantees supply sufficiency. In summary, with the presented approach, the cost-benefit equation of RhD determination on blood donations has been changed while supply sufficiency is maintained. Therefore, the debate on whether or not to molecularly screen donors for the *RHD* gene has been eliminated, at least in Switzerland.

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