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RHD PCR of D-Negative Blood Donors

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Summary
RHD PCR of blood donors may be used to reveal weak D, partial D, DEL and chimeric D+/D– donors among presumed D-negative blood donors. Units donated by such donors pose a definite yet low risk for anti-D immunization of transfusion recipients. The frequency of DEL donors among D-negative donors is 1:350 to 1:2,000 in Europe and up to 1:5 in Asian countries. Different strategies for *RHD* PCR of blood donors have been used. Probably, the most cost-efficient implementation is replacement of sensitive D antigen testing with the indirect antiglobulin test by *RHD* PCR in pools which might even reduce total testing cost.

Introduction

Soon after the identification of the RH genes, it became apparent that D-positive individuals have two different RH genes, while D-negatives generally only possess one [1]. The additional RH gene present in D-positives was characterized as *RHD* gene [2, 3] and its presence used to determine the D antigen status [4].

This clear-cut explanation of the D-positive / D-negative antigen status held until 1997, when reports appeared showing that the dictum '*RHD*-positive equals D-positive' was not always true for people of African [5] or Japanese [6] descent. Furthermore, even in a European population, alternative molecular bases for the D-negative phenotype were described [7].

Triggered by these observations, we aimed to determine the molecular bases of D-negative *RHD*-positive haplotypes in South-Western Germany on a larger scale [8]. For this sake, donors believed to be D-negative were checked by *RHD* PCR specific for the *RHD* promoter, *RHD* intron 4, *RHD* exon 7, and *RHD* exon 10. As part of the serologic work-up, the D-negative status was re-evaluated by the indirect antiglobulin test and adsorption/elution testing. Apart from a large range of different *RHD*-positive D-negative donors, surprisingly a relevant number of donors were D-positive in serologic re-evaluation: Among 754 C- or E-positive donors, 15 DEL and 4 weak or partial D donors were found, and among 7,688 ccddee donors, one chimeric D-positive/ D-negative donor with about 5% D-positive red blood cells (RBC) was detected. A look-back study of his prior donations indicated that both identified D-negative recipients had developed an anti-D. This observation raised the question whether D-negative donors should be checked by *RHD* PCR to exclude weak D-positive and DEL donors missed by serology.

A General View on Anti-D Immunization

When considering the assumed low immunogenicity of DEL units or comparing the frequency of anti-D immunization by DEL to the often higher frequency of other alloimmunization events [9], two facts on anti-D immunization should be realized:

– First, a prospective evaluation of anti-D immunization by weak D and DEL is difficult. Even after transfusion of normal D-positive units, only about 21–31% of patients develop an anti-D [10–12]. Weak D and DEL units are likely considerable to be less immunogenic. If no event is observed among n observations, the upper limit of the confidence interval for the frequency of the event is about 2,996/n [13]. Therefore, a study to exclude an immunogenicity of 2.5% (one tenth of normal D-positive units) would have to include 120 patients. This study size has never been reached in prospective studies [14] or look-back studies [8, 9, 15].

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– Second, a comparison with immunizations to other RBC antigens may be misleading. Anti-D is the most important cause of hemolytic disease of the newborn. Before the advent of anti-D prophylaxis, this disease occurred in a frequency of up to 1:23 among Rh-negative women [16]. Rh prophylaxis does not work in pre-immunized women. Therefore, even a low-titer anti-D immunization caused by a DEL unit will prevent anti-D prophylaxis from working and thus trigger the devastating cascade of ever increasing anti-D strength with each pregnancy. This mechanism is unique to antigen D, as no prophylaxis is used for the other RBC antigens, and antibodies to other antigens much rarer cause similar harm as a high-titer anti-D.

Evidence for Anti-D Immunization by Weak D Missed in Donor Testing

The immunogenicity of weak D has been questioned [17, 18] as only a few report of this event have been published: None of 49 D-negative recipients of 68 'Du' units developed an anti-D [14].

However, there is little doubt that transfusion of units donated by weak D donors can cause anti-D immunization in D-negative recipients: Even before the elucidation of the molecular basis of weak D, three anti-D immunization events by 'weak D', including a primary immunization by a unit with 800–1,500 antigens/cell, have been documented [19]. These immunizations occurred within less than 2 years when testing for weak D antigens was dropped in the Netherlands [20]. Whereas the underlying alleles of these donors were not evaluated and it might be argued that they represented exceptions carrying special alleles, reports of anti-D immunizations by units from donors with frequent weak D alleles appeared: In New Zealand, a ccD.Ee weak D type 2 donor with about 450 antigens/cell caused a primary anti-D immunization [21]. The same was shown for a weak D type 1 donor with *Cde in trans* further suppressing his antigen density to 357 antigens/cell [22]. In addition, an anti-D immunization event by the rare weak D type 26 was caused by a donor with an antigen density as low as 70 antigens/cell [23]. Considering the low likelihood of a look-back study in an inadvertently anti-D-immunized patient, these case reports are likely only the tip of the iceberg.

Evidence for Anti-D Immunization by DEL Donors

Evidence for anti-D immunization by DEL donors exists, although the risk may be considerably lower than for normal D: A female Austrian patient with an unexplained anti-D immunization was shown to have received a DEL unit harboring the rare DEL allele *RHD*(IVS5-38 del4) [24]. Shortly afterwards a considerable increase of the anti-D titer in a pre-immunized 67-year-old Japanese woman receiving two units with the DEL *RHD*(K409K) was observed [25]. This observation was important, since *RHD*(K409K) is the by far most frequent DEL allele in Asians and worldwide. [26]. Four years later, the same allele was shown to cause an anti-D immunization in a 69-year-old Korean male [27]. Still, anti-D immunizations caused by DEL donors seem to be rare events: Only one possible anti-D immunization event was found in a follow-up of 13 units from DEL donors in Denmark [9], and only 3 of 82 D-negative recipients of *RHD*(93insT) units had developed an anti-D in Canada [15]. In both studies, transfusion of D-positive platelet units was an alternative explanation for the anti-D immunization events, and the immunogenicity of DEL seemed to be lower than that of many other blood group antigens.

Evidence for Anti-D Immunization by RHD+/– Chimeric Donors

The evidence of anti-D immunization by the RHD+/– chimeric donor in the seminal study [8] was convincing, as both identified recipients who were D-negative and not known to possess an anti-D prior to the transfusion had an anti-D after the transfusion. Furthermore, a quick calculation of the number of D-positive RBC revealed that this observation was far from surprising: An RBC unit contains about 220 ml of packed RBC, 5% of which equals 11 ml D-positive RBC. This volume is much higher than the usual volume of D-positive fetal RBC entering the maternal circulation during delivery, a known cause of anti-D immunization. Although the evidence of immunization is obvious, it should be realized that no second D-positive / D-negative chimeric donor was identified in follow-up studies [28 -31], and this event thus seems to be a rarity.

Frequency and Type of Seemingly D-Negative, *RHD*-Positive Donors

Currently, in the RhesusBase [32] 30 alleles are listed that have been described to be associated with a DEL phenotype (table 1). As the borderline between weak D and DEL is not defined and may vary between laboratories depending on the sensitivity of the indirect antiglobulin test used, some of these alleles may represent weak D with low antigen density. In addition, weak D with low antigen D expression like weak D type 32 [30] or weak partial D like D category VI type I may appear as DEL if a *Cde* allele is in trans. On the other hand, false-positive results in adsorption/elution technique may also occur [35]; so some alleles observed only once and described as DEL might rather represent D-negative alleles.

Two main mechanisms may cause a DEL phenotype:

– Classically, a mutation near the splice site hampers normal splicing of *RHD*. This mechanism was well studied for *RHD*(K409K), in which a 'silent' 1227G>A substitution in codon 409 is immediately adjacent to the exon 9 / intron 9

Table 1. Alleles for which a DEL phenotype has been described [32]

Allele (Trivial name)	Structure	Haplo-type	Mechanism	Phenotype	Distribution*	References	Comments
RHD(M1I)	*RHD*(3G>A)	Variable	loss of start codon	DEL	R (China, Germany)	[33]	
RHD(R10W) (Weak D type 61)	*RHD*(28C>T)	CDe	MIS	DEL, weak D	R	[33]	DEL phenotype if categorized by tube testing; independently characterized as weak D AM412754
RHD(W16R)	*RHD*(46T>C)	cDE	MIS	DEL	S(Switzerland)	HE999546	
RHD(W16X)	*RHD*(48G>A)	CDe	STOP in exon 1	D-negative, DEL	R (Germany)	[8]	reported as DEL (same donor tested on three donations as DEL) in a later study [35]
RHD(L18P)	*RHD*(53T>C)		MIS	DEL	S (China)	[33]	
RHD(93insT)	*RHD*(93insT)	CDe	FS in exon 1	DEL	R(Germany, Denmark, Spain)	[34]	DEL status reported in follow-up study[28]
RHD(L38X)	*RHD*(113T>A)		STOP in exon 1	DEL?	S (Germany)	[35]	
RHD(147delA)	*RHD*(147delA, IVS1+6delA)	CDe	FS in exon 1	DEL	S (Germany)	[28]	
RHD(IVS1+1G>A)	*RHD*(IVS1+1G>A)		SPL	DEL	S (Japan)	[36]	
RHD(IVS1-29G>C)	*RHD*(IVS1-29G>C)	CDe	SPL	DEL	S (Poland)	HE971139	this allele was initially reported as *RHD*(IVS2-29G>C)
RHD(L84P)	*RHD*(251T>C)		MIS	DEL	S (China)	[33]	comparably high antigen density
RHD(IVS2-2A>G)	*RHD*(IVS2-2A>G)	CDe	SPL	DEL	S (Denmark)	[9]	
RHD(S112T)	*RHD*(IVS1-29G>C, 335G>C)		SPL	DEL	S (Switzerland)	HE999547	both mutations affect the splice site
RHD(A137E)	*RHD*(410C>A)	CDe	MIS	DEL	S (China)	[37]	DEL status according to tube IAT
RHD(L153P)	*RHD*(458T>C)	cDE	MIS	DEL	S (Germany)	[28]	
RHD(IVS3+1G>A)	*RHD*(IVS3+1G>A)	CDe	SPL	DEL / D-negative / Partial DEL	R (Germany, Denmark, Poland, Austria)	[8]	this allele is almost D-negative
RHD(G212R)	*RHD*(634G>C)	cDe	missense mutation / splice site affected	DEL	S (Germany)	[28]	
RHD(IVS5-38del TCTC)	*RHD*(IVS5-38del TCTC)	CDe	unknown	DEL	S (Austria)	[24]	the IVS5–38 del TCTC polymorphism is also found in *RHD* alleles with normal antigen strength and therefore not causative [38]
RHD(P291R)	*RHD*(872C>G)	CDe	MIS	DEL	S (Switzerland)	HE999545	the same missense mutation occurs in the DEL weak D type 4.3

Table 1 continued on next page

Table 1. Continued

Allele (Trivial name)	Structure	Haplo-type	Mechanism	Phenotype	Distribution*	References	Comments
RHD(T201R, F223V, P291R) (weak D type 4.3)	*RHD*(602C>G,667T >G,819G >A,872C>G)	cDe	MIS	DEL	R (Austria, Germany)	[39]	the DEL phenotype is probably caused by the P291R substitution
RHD(M295I)	*RHD*(885G>T)	CDe	MIS	DEL / weak D	R (Germany)	[8]	borderline DEL / weak D. The same mutation in a cDe haplotype causes the weak D type 11 phenotype.
RHD(IVS8-31 T>C)	*RHD*(IVS8-31 T>C)	CDe	SPL	D-negative / DEL / weak D	R (Germany)	[35]	
RHD(Y401X)	*RHD*(1203T>A)	cDE	STOP near 3' end	DEL D-negative	R	[23]	initially described as D-negative, reported as DEL in another study [28]
RHD(D404H)	*RHD*(1210G>C)	cDE	MIS	DEL	S (Portugal)	JX114749	
RHD(W408R)	*RHD*(1222T>C)	CDe	MIS	DEL	S (Korea)	[40]	
RHD(K409K)	*RHD*(1227G>A)	CDe	SPL	DEL	Most frequent DEL in Asia [26[	[8]	this allele has initially been misinterpreted as *RHD*(delEx9)
RHD(X418L)	*RHD*(1252ins T)	CDe	loss of STOP	DEL	R (Austria, Germany)	[23]	
RHD-RHCE(2–5)-RHD	*RHD-RHCE(2–5)-RHD*	CDe?	hybrid allele	Partial D, DEL	S (China)	[33]	the predicted structure is similar to DVI type IV, a partial D frequent in Spain [41]. The reason for the different phenotype is unknown.
RHD-RHCE(4–9)-RHD	*RHD-RHCE(4–9)-RHD*		large hybrid	D-negative, DEL	R (China)	[37]	this allele was initially characterized as D-negative but appeared as DEL in one study [37]
DBU	*RHD-RHCE(5–7)-RHD*	cDE	loss of D-specific exofacial loops	DEL, Expected partial D	S (Germany)	[28]	
RHD(delEx8)	*RHD*(delEx8)	CDe	995 bp deletion including exon 8	DEL	one pedigree (Libanese)	[42]	
RHD(delEx9) (obsolete)							the observations of this allele represent *RHD*(K409K) [43]
RHD(delEx10)						[44]	
RHD-RHCE(10) (obsolete?)	*RHD-RHCE*(10)					[37]	the observation of this allele likely represent *RHD*(delEx10)

MIS = Missense mutation; STOP = stop codon; SPL = splice site mutation; FS = frameshift; S = single observation; R = repeatedly observed.

boundary. Most transcripts of this DEL lack RHD exon 9 [45]. It is unknown, whether the faint residual D antigen expression is due to D antigen expression by misspliced transcripts or a very low number of correctly spliced transcripts. A similar mechanism is likely to work in *RHD*(IVS3+1G>A) that in addition seems to lack some specific D epitopes. Although it might be reasoned that the partial D character might be due to a predominance of misspliced transcripts, the exact mechanism is still unknown.

- Some missense mutations have so much impact on the correct expression of RhD so that a DEL phenotype results. Often, these alleles have a borderline weak D / DEL antigen density. An interesting example is *RHD*(M295I), which displays the weak D type 11 phenotype when it occurs in a cDe haplotype [46] and a DEL phenotype when it occurs in a CDe haplotype [8].

In addition, there is a flurry of different rare mechanisms:

- Some mutations generally expected to destroy any production of intact RhD protein are associated with a DEL phenotype. The most important example is *RHD*(93insT) which shows a DEL phenotype despite a frameshift. For this allele, it was argued that transcription slipping might result in a low number of wild-type transcripts. Considering the location of this mutation in exon 1 and the DEL phenotype of *RHD*(M1I) [33] lacking the normal start codon, another explanation is the use of an alternative start codon.
- In *RHD*(X418L), the stop codon is destroyed resulting in a predicted structure with 71 additional tailing amino acids [23].

A few DEL are characterized by polymorphisms for which it is unknown whether and why they cause the DEL phenotype. For example, one DEL was shown to be associated with an IVS5-38 del4 polymorphism [24], but in a later observation the same polymorphism was observed in a variety of *RHD* alleles of normal antigen strength [38].

Which *RHD* PCR Should Be Used?

The use of *RHD* PCR to detect weak D and DEL donors serologically mistyped as D-negative is a task considerably different from other uses of *RHD* PCR: A very large number of samples have to be tested, imposing considerable pressure on cost and effort. In contrast, only a few positive samples are found, making accessory testing to fully characterize these samples feasible. Therefore, often a two-stage strategy is used that consists of 'screening detection' of any *RHD*-positive sample followed by a more in-depth characterization of only these samples.

For conventional applications, an *RHD* PCR should rely on testing at least two different *RHD*-specific polymorphisms, because otherwise hybrid *RHD-RHCE-RHD* alleles expressing partial D phenotype may be missed. Since most DEL and weak D differ from normal *RHD* by splice site or missense mutations, they are not affected by this problem, and testing for almost any *RHD*-specific polymorphism will reveal the vast majority of DEL samples. Therefore, if sensitive serologic testing is continued to detect partial D caused by hybrid alleles, testing a single *RHD* polymorphism by PCR may be a possible choice to find the DEL samples [28, 35, 47].

Another difference to conventional *RHD* PCR applications is the possibility to start with pooled testing [8, 28, 30, 35, 39, 48]: As long as the samples have initially be checked at least by direct agglutination with anti-D, in Caucasian populations only a minority of samples will be *RHD*-positive.

While almost any *RHD* PCR will be sensitive to detect DEL, obtaining specificity is more difficult. Most *RHD* PCR testing for DEL will also reveal *RHD*-positive alleles not expressing any D antigen ('D-negative *RHD*-positive alleles'), like those with stop codons. It is nearly impossible to devise a PCR system that will not give a positive result for these alleles, and further characterization of the samples by extensive molecular or serologic testing may be necessary. However, the choice of the polymorphisms used for initial testing may help to reduce the rate of D-negative *RHD*-positive samples detected:

- Generally, large *RHD-CE-D* hybrids in which at least *RHD* exon 4–7 is substituted by *RHCE* are D-negative. Therefore, using a polymorphism inside this region will avoid detecting these rather frequent D-negative, *RHD*-positive hybrids.
- In a population with a relevant admixture of individuals of African descent, a relevant number of D-negatives will be *RHDψ* positive. In such a population, it is advantageous to devise the initial PCR in a way that it does not detect *RHDψ*.

If an *RHD* PCR approach is used that restricts initial testing to a polymorphism within the range of *RHD* exon 4–7, about one third to one half of the detected donors carry these D-negative *RHD*-positive alleles [28, 31] and could thus be kept in the D-negative donor pool.

D-Positive Alleles Missed by PCR

Despite the simplicity to detect most DEL, it must be realized that some alleles encoding for D epitope expression are prone to be missed, especially if the initial 'screening' *RHD* PCR is reduced to a single or a few *RHD*-specific polymorphisms:

- In almost any approach not specifically devised to detect these alleles, *RHCE* alleles expressing D epitopes due to mutations unrelated to *RHD* will be missed. The most pertinent of these alleles are ceRT [49] and ceSL [50].
- Likewise, *RHCE* alleles expressing D epitopes due to the presence of only one *RHD*-specific nucleotide like ceCF [51] are missed, if this D-specific polymorphism is not tested for. To a lesser extent, this problem applies to *RHCE* alleles with short stretches of *RHD* included, like DHAR in which only exon 5 displays *RHD* sequence.
- If a single-polymorphism testing strategy is used, short hybrid alleles, generally associated with a partial D phenotype, are missed. For example, testing exon 7 [35] misses DBU,

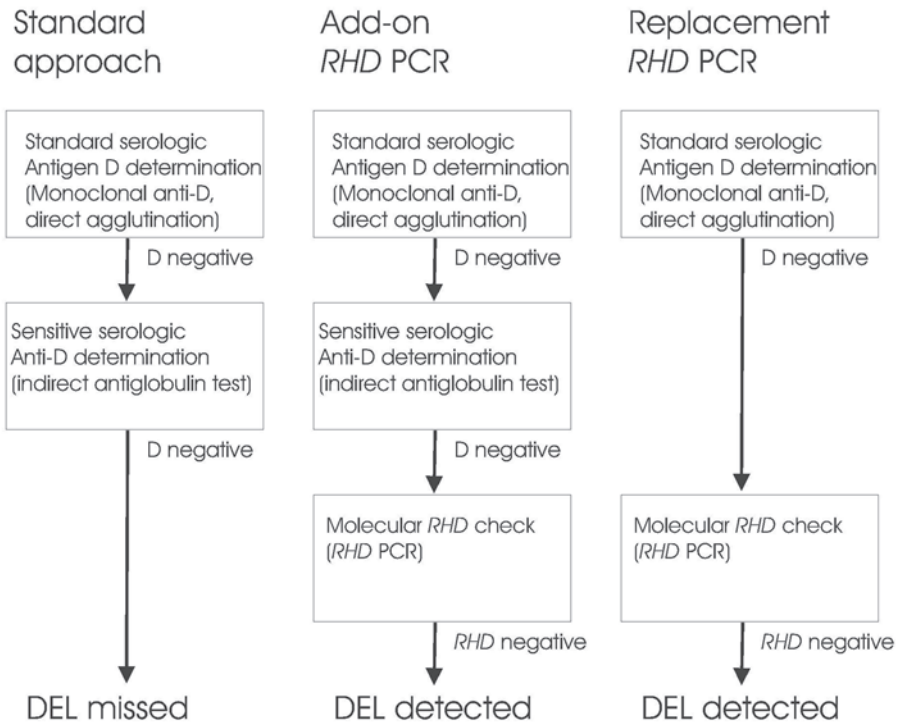

Fig. 1. Add-on and replacement *RHD* PCR. Different approaches for the determination of the antigen D status of first time donors are shown. **Left:** In standard antigen D determination, donors are first tested with a directly agglutinating monoclonal anti-D, and D-negative donors are then checked by the indirect agglutinin test with an oligoclonal anti-D to detect weak D and partial D antigens. DEL are missed by this approach. **Middle:** DEL may be detected by adding *RHD* PCR to the standard D determination. This approach eliminates any risk that D-positive donors detectable by the serologic approach are missed, but of cause the cost must be higher than that of the conventional approach. **Right:** DEL may be also detected by testing donors D-negative by direct agglutination by *RHD* PCR. This approach is safe, as long as the *RHD* PCR is devised in a way that weak D and partial D alleles are detected, too. The cost of this approach may be higher or lower than that of the standard approach, depending on the relative cost of the indirect antiglobulin test and the *RHD* PCR used.

DBT, and many DIV variants; testing intron 4 [28] will miss DVI variants. The relevance of such misses depends on the serologic characteristics of these variants, for example, most DIV variants are readily detected by direct agglutination while DBU represents a DEL phenotype.

Frequency of Missed D-Positive Samples

A survey of published population studies of serologically D-negative donors is given in table 2. Generally, the frequency of DEL among Europeans is 1:350 to 1:2,000, while it gets as high as 1:5 among Asians. It should be noted that in many studies [8, 23, 47, 56] weak D or partial D donors were detected among donors previously believed to be D-negative.

Possible Testing Strategies

Different blood services introduced *RHD* PCR for seemingly D-negative blood donors; however, approaches vary. A few considerations will be detailed below.

First-Time Donors versus Repeat Donors

From a logistic point of view, testing repeat donors is much more complicated than testing first-time donors: i) Generally, the sheer number of repeat donors is much higher than those of first-time donors. ii) In order to prevent the repeat testing of the donors, it is necessary to implement a strategy to mark the donors as tested. iii) Often, pooled platelet units are produced exclusively from repeat donors. *RHD* PCR of those donors will hamper the delivery of the units or necessitate the delivery before D antigen determination is finished.

Based on these considerations and a perceived low cost-efficiency of the *RHD* PCR, several blood services started by testing exclusively first-time donors. After many years, such strategy should result in a D-negative donor pool largely tested by *RHD* PCR. However, many donors continue to donate for decades, and the most pertinent examples of missed weak D donors were 'old-time' donors [8, 23], who had been tested for weak D many decades ago when the sera were less potent than today and only tube testing was available. Therefore, testing of all donors is recommended, once the logistic basis to exclude repeated testing and delays in platelet unit delivery is established.

C/E-Positive Donors versus All Donors

In many populations, the frequency of DEL among *CDe* and *cDE* haplotypes is much higher than among *cDe* haplotypes. For example, in 6 years *RHD* PCR in South-Western Germany [28], the DEL frequency among Ccddee donors was 1:51, among ccddEe donors 1:344, among all C or E positive donors 1:67, and among ccddee donors only 1:43,053, more than 600 times lower than among those with C or E. Since the cost per detected donor depends on the number of donors that need to be tested in order to identify one DEL, weak D or chimeric donor, focusing on donors with C or E is much more cost-efficient than testing all donors. On the other hand, in some countries, like Germany, premenopausal women are exclusively supplied with units compatible for all Rh antigens. A ccDELee unit can possibly be transfused to any D-negative premenopausal patient, while only 4% of all premenopausal women are eligible for Ccddee units. Hence, the probability of a ccDELee unit

Table 2. Population studies of DEL

Population	RHD exons screened	D-negative donors screened	DEL detected	Frequency of DEL among D-negative	Most frequent alleles	Number of different DEL alleles found
Brazil [52]	intron 4, exon 10	239	0	<1:80		
Brazil [48]	intron 4, exon 7, pools of 10	2,450	19 (including weak D)	1:129		
Central Europe [23], donors with C or E	5′ UTR, 3, 10	1,700	15 DEL + 5 weak D / partial D	1:113	*RHD*(M295I), *RHD*(IVS3+1G>A)	*3*
China [53]	intron 4, exon 7, (5'UTR, exon 10)	155 (negative on direct agglutination)	31 (+3 weak D)	1:5	*RHD*(K409K)	*1*
China (Shanghai) [37]	(serologic screening)	1,585	279	1:6	*RHD*(K409K)	
Denmark [9]	5.7.8	5,058 (4,932 results available)	2*	1:2,029		2
Denmark [47], donors with C or E	exon 10	233	3 DEL + 1 weak D	1:78		*3*
Germany(South-West) [8], donors with C or E	5′ UTR, intron 4, exons 7, 10	754	15 DEL + 4 weak D / partial D	1:50	*RHD*(M295I), *RHD*(K409K)	*3*
Germany(South-West) [8], ccddee donors	5′ UTR, intron 4, exon 10	7,688	1 chimeric donor	<1:2,500		
Germany (South-West) [28]	intron 4 (pools of 20)	46,133	47	1:982	*RHD*(IVS3+1G>A), *RHD*(M295I)	*10*
Germany (North) [31, 35]	exon 7 (pools of up to 40)	46,756	76	1:615	*RHD*(IVS3+1G>A), *RHD*(M295I)	*15*
Italy [54]	commercial PCR systems, pools of 5	235	1	1:235	*RHD*(M295I)	*1*
Korea [43]	intron 4, exon 7	126	16 + weak D	1:8	*RHD*(K409K)	*1*
Korea [40]	3.5.7.10 plus intron 4	264	43	1:6	*RHD*(K409K)	
Upper Austria [39]	4.7.10 in pools of 20	2,427	3	1:809	*RHD*(IVS3+1G>A)	*2*
Upper Austria [30]	4.7.10 in pools of 20	23,330	66	1:353	*weak D type 4.3, RHD*(IVS3+1G>A)	*6*
Thailand [55]	(serologic screening)	254	50	1:5	*RHD*(K409K)	
Tunisia [56]		488	3 weak D, 1 partial D (missed despite IAT)			

*In addition, an *RHD*(IVS3+1G>A) sample was detected but characterized as D-negative.

to be transfused to a premenopausal woman is about 25 times larger than that of a Ccddee unit, partly counteracting the increased probability of a DEL unit among Ccddee. Even considering this fact, the cost-efficiency regarding prevention of DEL transfusion to D-negative premenopausal women is about 37 times higher if only donors with C or E are tested.

In conclusion, testing donors with a C or E is considerably more cost-efficient than testing all D-negative donors. However, if the risk of immunization by DEL, weak D, or chimeric donors shall be abolished, testing all donors is necessary.

Add-on Testing versus Replacement of Indirect Antiglobulin Test

Current D antigen determination for blood donors often includes sensitive testing for the first or first few donations followed by confirmatory testing by direct agglutination only. If the *RHD* PCR is added to such testing regime, there is no risk that the new strategy misses donors that would have been detected by the old serologic strategy. Hence, such 'add-on' testing may be realized with very simple and cheap *RHD* PCR approaches. However, the total cost of D antigen determination is always higher than that of a serologic testing strategy.

An alternative approach consists of replacement of the sensitive serologic testing by *RHD* PCR. In this approach, serologic donor testing is reduced to direct agglutination, and all first-time donors negative by direct agglutination are checked by *RHD* PCR (fig. 1). Obviously, in this setting the *RHD* PCR must be devised in a form to assure that any relevant D-positive *RHD* variant that may be missed by direct agglutination is detected. The major advantage of this approach is the elimination of the non-negligible testing cost for D antigen determination in the indirect antiglobulin test.

Single Donor Testing versus Pooled RHD PCR

As *RHD*-positive donors are rare among seeming D-negative Caucasian donors, many blood services use pooled *RHD* PCR. Of course, in populations with a relevant number of individuals of African or Asian descent, *RHD*-positive D-negative alleles are more frequent, and pooled testing is only advisable if the PCR is devised in a way that the frequent *RHD*-positive D-negative allele of the population is not detected. A possible pitfall of pooling may be the varying DNA content of different donors units due to different leucocyte concentration. However, most PCR systems are devised in a way that even very small contaminations with D-positive DNA may be detected. It should be remembered that in the initial study on this topic, a donor with only 5% D-positive RBC was found with pooled testing [8].

Further Work-Up of *RHD*-Positive Units

A drawback of the *RHD* PCR is the fact that D-negative *RHD*-positive alleles are detected as well. If these donations shall not be moved to the D-positive donor pool, further work-up is necessary. Serologic analysis of these samples is time-consuming and may be unreliable [31]. From a cost-efficiency perspective, using all *RHD*-positive units exclusively for D-positive recipients may be a rational choice. Such measure would reduce the D-negative donor pool by about 0.1% but save any additional testing cost.

If the most frequent D-negative alleles of the population are known, donors carrying these alleles can be identified by PCR specifically detecting these alleles; the rare unclassifiable samples might be further analyzed by sequencing. Since this work-up may be very costly, these added costs should not be neglected when switching to *RHD* PCR is considered. However, these costs will only occur once an *RHD* PCR-positive donor is detected, reducing their impact on general donor testing. For example, assuming that donors are tested in pools of 20 with a PCR assay at EUR6.00 per PCR, 1 of 1,000 donors is *RHD*-positive and further work-up is EUR 80.00 per *RHD*-positive donation, PCR cost per presumed D-negative donation will be EUR 0.30 and work-up cost per presumed D-negative donation EUR 0.08.

Examples of *RHD* PCR Programs

A survey of current *RHD* PCR programs is given in table 3. Obviously, *RHD* PCR is increasingly used, but approaches still vary. The first country in which *RHD* PCR has become obligatory is Switzerland.

Cost-Efficiency Considerations

Given the low risk of anti-D immunization by transfusion of DEL units, the cost-efficiency of *RHD* PCR for blood donors has been disputed. For example, in a recent review, it was concluded that 'For routine ABO and D determination, DNA testing is more time-consuming, more expensive, prone to misinterpretation, and thus, not an improvement over hemagglutination' [57]. Westhoff [58] argued that focusing on D would be inappropriate as long as immunization to K and c would be considered irrelevant in premenopausal women.

The current standard in several European countries, like Germany, includes sensitive D antigen testing of donors and matching for K and c in premenopausal women, and it is unlikely that this standard will be reduced in the near future. Thus, cost-efficiency can be reduced to the question whether using *RHD* PCR is cost-efficient compared to standard sensitive D antigen testing with indirect antiglobulin test.

As outlined above, the cost-efficiency and imposed workload of *RHD* PCR for blood donors considerably depends on the approach chosen. The by far most cost-efficient approach is replacement of indirect antiglobulin test testing by *RHD* PCR and using all *RHD*-positive units for D-positive recipients. In this approach, workload and testing cost is likely comparable to the 'old' serologic approach and it may be anticipated that with on-

Table 3. Routine *RHD* PCR of blood donors

Blood Service	Testing strategy
Red Cross Blood Service Baden Württemberg – Hesse [28] (Germany)	all first time donors, pooled testing for *RHD* intron 4
Red Cross Transfusion Service of Upper Austria [30]	all first time donors, pooled testing for *RHD* intron 4.7.10; no IAT
NIH (USA)	single donor testing (WA Flegel, Washington, personal communication)
Aarhus Hospital (Denmark) [47]	exon 10 for donors with C or E
Red Cross Blood Service NSTOB (Germany)	all first time donors, pooled testing for *RHD* exon 7
Albert Einstein Hospital, São Paulo (Brazil) [48]	
Red Cross Blood Service (Bern, Switzerland)*	pooled testing for exons 3.5.10 (H Hustinx, Bern, personal communication)
Red Cross Blood Service (Zurich, Switzerland)*	pooled testing intron 4 / exons 5 +7 (until 2011), single donor exon 5, 7 and 3' untranslated (since 2012) (C Gassner, Zurich, personal communication)
Red Cross Blood Service (Innsbruck, Austria)	single donor testing of donors with C or E

*RHD PCR of D-negative donors is mandatory in Switzerland since January 2013, serologic testing by IAT has been dropped.

going reduction of PCR testing cost, this approach may become – or even may already be – cheaper than a merely serologic testing. Hence, I expect that in the end, indirect antiglobulin testing will be replaced by *RHD* PCR, not because missing DEL is so risky, but because *RHD* PCR will become the cheaper way to detect weak D, partial D, and DEL, making any cost-efficiency discussions absurd.

Conclusion

It is obvious that *RHD* PCR of blood donors may prevent anti-D immunizations and will, albeit rarely, prevent the dismal spiral of ever increasing anti-D in anti-D-immunized pregnant women. Furthermore, *RHD* PCR may become cheaper than testing donors with the indirect antiglobulin test. For those countries like Germany that decided to go with sensitive testing of donors for the D antigen, the switch from the indirect antiglobulin test to *RHD* PCR would be an example of a change in testing strategies in which the superior testing strategy ultimately turns out to be the cheaper one.

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The author receives royalties as inventor of patents on the molecular genetics of the RH blood group

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