*Original article*

# Serologically D-negative blood donors in Thailand: molecular variants and diagnostic strategy

Pornlada Nuchnoi[1,2], Jairak Thongbut[3], Caroline Bénech[4,5], Pawinee Kupatawintu[3], Dootchai Chaiwanichsiri[3], Claude Férec[4], Yann Fichou[4,5]

[1]*Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand;*
[2]*Center of Research and Innovation, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand;*
[3]*National Blood Centre, Thai Red Cross Society, Bangkok, Thailand;*
[4]*Univ. Brest, Inserm, EFS, UMR1078, GGB, Brest, France;*
[5]*Laboratory of Excellence GR-Ex, Paris, France*

**Background** -Discriminating individuals with "Asian type DEL" from those who are "true D-negative" (D–) among serologically D– donors/patients in Asia would be very valuable, as clinical outcomes are different in these groups. Here we investigated the molecular basis of D-negativity in Thai blood donors, designing a specific strategy for this purpose.

**Materials and methods** - After routine testing, a total of 1,270 serologically D– blood donors originating from Central, Northeastern and South Thailand underwent analysis of the *RHD* gene by (i) quantitative multiplex polymerase chain reaction of short fluorescent fragments (QMPSF); (ii) direct sequencing of exon 9 to identify the c.1227G>A variant defining the Asian type *DEL* allele; and (iii) direct sequencing of the other exons.

**Results** - The most common observation was whole deletion of the gene (i.e. *RHD*01N.01*; allele frequency: 86.81%), followed by the Asian type *DEL* allele (*RHD*01EL.01*; 7.60%) and a D-negative hybrid allele (*RHD*01N.03*; 3.46%). Four novel alleles, including one with a 13.1 kilobase-deletion, were identified and characterized. All but one *RHD*01EL.01* allele carriers (183/184) were C-positive (C+), suggesting that this latter subset may be screened specifically when investigating the c.1227G>A variant, which can be identified with 100% accuracy by a specific Tm-shift genotyping assay.

**Discussion** - On the basis of our extensive molecular findings, we have designed a dedicated diagnostic strategy based on Rh C antigen typing followed by a genotyping test. Implementation of this method in all or selected groups of serologically D– donors/patients will contribute to improve the management of transfusion and pregnancy in Thailand.

**Keywords:** *Asia, genotyping, Rh system, RHD gene, variant.*

## INTRODUCTION

Expression of the D antigen in the Rh blood group system (ISBT004) is routinely assessed by serological testing using panels of monoclonal antibodies. On the basis of the presence and absence of the antigen at the surface of red blood cells (RBC), donors/patients are classified as D-positive (D+) and D-negative (D–), respectively. The prevalence of D antigen expression has long been known to be dependent on

Arrived: 4 July 2022
Revision accepted: 3 October 2022
**Correspondence:** Yann Fichou
e-mail: : Yann.Fichou@efs.sante.fr

the origin of the population(s) of interest. In East and Southeast Asia, more than 99% of the general population are D+[1-4]. In the remaining apparent (or serologically) D– subset, a variant *RHD* allele harboring the c.1227G>A single nucleotide variant (SNV) has been reported in 10-30% individuals[5-8]. This synonymous SNV is located at the final nucleotide position in *RHD* exon 9[9], and has been shown to alter cellular splicing dramatically, mostly resulting in the production of nonfunctional, aberrant transcript isoforms[10-13]. This so-called Asian type *DEL* allele (*RHD*01EL.01* or *RHD*DEL1*, according to the official ISBT nomenclature; URL: www.isbtweb.org/resource/004rhd.html) confers a DEL phenotype[5,9], consisting of the expression of minute amounts of the D antigen which can be detected specifically by an adsorption-elution test[14]. Importantly, Asian type DEL RBCs are thought to express the full repertoire of D epitopes[15]. It was thus proposed that such patients could be transfused with D+ RBC with no risk of alloimmunization[15], as supported by further clinical observations[16-19], thereby contributing to restrict the provision of precious, limited D– RBCs strictly to "true" D– patients. Such protocols have not been implemented to date, but discriminating DEL RBCs from true D– RBCs by a simple selective method would be very valuable in order to optimize RBC resources.

In Thailand, we recently showed that 15.6% of serologically D– Thai blood donors carry the Asian type *DEL* allele in a comprehensive, large-scale study[8]. On the basis of our findings, it seemed that it could be a relevant part of the global Thai transfusion policy to implement a dedicated strategy to identify those donors specifically. Furthermore, because we found several novel alleles in our previous studies[8,20], we decided to investigate *RHD* genetic polymorphism further in the Thai population. Here, we (i) describe the nature and frequency of the *RHD* allele in 1,270 newly genotyped, serologically D– Thai blood donors, (ii) report the novel alleles identified in the study, (iii) describe the design and validation of a simple genotyping approach to screen for the c.1227G>A SNV rapidly in order to distinguish the Asian DEL samples from the true D– samples, and (iv) propose a global strategy for managing serologically D– donors and patients to be implemented in the Thai healthcare system.

## MATERIALS AND METHODS

### Blood samples and serological testing

The study was approved by the Research Ethics Committee, National Blood Center (NBC), Thai Red Cross Society (COA No. NBC 13/2018) for human research subjects. Blood donors were categorized into three groups according to their geographical origin in Thailand: (i) Central region (NBC, Bangkok); (ii) Northeastern region (Regional Blood Centers: Khon Kaen, Ubon Ratchathani, and Nakhon Ratchasima provinces); and (iii) Southern region (Regional Blood Centers: Nakhon Si Thammarat, Songkhla, and Phuket provinces). The Rh typing procedure (D, C, c, E, e) was carried out as described previously[8] (details about the routine D typing procedure are provided in the *Online Supplementary Content*, **Table SI**). All samples yielding a D–or weak D reaction on initial testing were further analyzed at the NBC (Bangkok) by the indirect antiglobulin test (IAT) with a standard tube method using two blended monoclonal IgM/IgG anti-D reagents: (i) IgM clone LDM2 and IgG clone LHM77 (NBC), and (ii) IgM clone RUM-1 and IgG clone MS-26 (Plasmatec, Bridport, UK).

### Genomic DNA extraction and molecular analysis of the *RH* genes

Genomic DNA was extracted from whole blood by a fully automated protocol (QIAsymphony DSP DNA kit in combination with the QIAsymphony SP machine, Qiagen, Valencia, Spain) as recommended by the manufacturer. DNA concentration and purity ($A_{260}/A_{280}$) were measured by spectrophotometry (NanoDrop One Spectrophotometer, Thermo Fisher Scientific, Illkirch-Graffenstaden, France). On the basis of our previous studies[8,20], genotyping of the serologically D– samples was first carried out by quantitative multiplex polymerase chain reaction (PCR) of short fluorescent fragments (QMPSF) to identify structural variants, including deletions, duplications and hybrid genes, as previously described[21]. Second, when QMPSF was inconclusive, *RHD* exon 9 was sequenced to identify the c.1227G>A variation defining the Asian *DEL* allele. Finally, if the c.1227G>A substitution was not detected, all *RHD* exons were sequenced to identify potential SNVs in conditions previously described[22].

Three and two genomic regions in *RHD* introns 3 and 6, respectively, were targeted by PCR amplification, to characterize novel alleles. The three primer pairs for marker amplifications in intron 3 were derived from a previous work[24] (RHD_i3a: 5'-CTCATCTGGCACAACTCAGCG-

3'/5'-CCAGATCTATCCCACCCCAACA-3' (73 bp); RHD_i3b: 5'-GGCTGACATCATCAGTGACCAAGA-3'/5'-CATCACACTCTCCCTTTCTTGCTGT-3' (70 bp); RHD_i3c: 5'-AATCCCCAAGTGTTCTTCCTGAAC-3'/5'-TAAGAACTGAAAAGCGGGCTTGT-3' [80 bp]), while two sets of primer pairs were newly designed in intron 6 (RHD_i6a (88 bp): 5'-AGCGACTCAGGAGGCTGAGACA-3'/5'-GCTGGAGTGCCATGGCACG-3'; RHD_i6b (169 bp): 5'-CATCTCAGCTTACTGCAAGCTCC-3'/5'-GTCGGGAGTTCAAGACCAGCAG-3'). PCR conditions were as described before[24].

### Asian type *DEL* genotyping assay

A Tm-shift assay targeting the c.1227 nucleotide position in *RHD* exon 9 was designed as previously described[22]. Briefly, real-time PCR amplification was carried out in a final volume of 10 µL with a PCR Master Mix (1X HotStarTaq PCR Master Mix, Qiagen), 0.3 µM RHD_1227G_F (c.1227G-specific forward primer: 5'-GC GGGCAGGGCGGCGATGACCAAGTTTTCTGGAAG-3'), 0.5 µM RHD_1227A_F (c.1227A-specific forward primer: 5'-GCGGGCGATGACCAAGTTTTCTGGAAA-3'), 0.5 µM RHD_e9misRb (reverse primer: 5'-CTCATAAACAGCAAG TCAACATATA**C**ACT-3'; a single mismatch is underlined in bold), 0.5 µM fluorescent dye (SYTO 9, Thermo Fisher Scientific), and 10-100 ng genomic DNA (gDNA) in a real-time PCR machine (LightCycler 480 II, Roche, Meylan, France). The conditions are: initial denaturation at 95°C for 15 min; followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 64°C for 10 sec, and extension at 72°C for 10 sec; denaturation at 95°C for 20 sec; and melting-curve analysis with fluorescent intensity measurement in a linear denaturation ramp from 65 to 95°C (0.06°C/sec; 10 acquisitions/sec).

## RESULTS

### Selection of the serologically D-negative Thai blood donors, prevalence of Rh C/E antigen expression and molecular investigation

Following automated routine testing, Thai blood donor samples typed as D– were tested by IAT. A total of 1,270 samples with a negative IAT were selected for subsequent genotyping of the *RHD* gene (Central Thailand: n=800; Northeastern Thailand: n=220; Southern Thailand: n=250). Of these, 598 (47.1%) expressed the C and/or E antigens (C/E+), while the other 672 (52.9%) were C/E-negative (C/E–).

Using our three-step molecular typing strategy, consisting of (i) *RHD/RHCE* QMPSF, (ii) *RHD* exon 9 sequencing and (iii) sequencing of all exons, 2,525 variant alleles were identified in the 1,270 serologically D– donors, while 15 alleles (in 14 samples) were found to be "wild-type", or conventional, *RHD*01* (**Table I**). As expected, the *RHD*01N.01* allele was by far the most common, followed by *RHD *01EL.01* and the *D*-negative *RHD *01N.03* allele (**Table I**).

Next, in order to get insights into the relationship between the *RHD* genotype and Rh CcEe phenotype, the samples and their respective genotypes were subcategorized as a function of Rh C and/or E antigen expression. Almost all C/E– samples had the **01N.01/*01N.01* genotype (670/672, 99.7%), while all 27 genotypes were found in the C/E+ subset (**Table II**). This observation indicates that expression of C and/or E in Thai blood donors is associated with a broader molecular variability in the *RHD* gene, which is an important result for any subsequent potential diagnostic strategy. The regional distribution of the samples, as well as their corresponding genotypes and C/E antigen expression status, showed no statistical difference (*data not shown*; $\chi^2$ test, p=0.1116).

**Table I** - *Allele frequency in 1,270 serologically D-negative Thai blood donor samples*

| Allele[1] | Occurence (%) | |
|---|---|---|
| ****01N.01*** | 2,205 | (86.81) |
| ****01EL.01*** | 193 | (7.60) |
| ****01N.03*** | 88 | (3.46) |
| ****D-CE(3-10)*** | 15 | (0.59) |
| ****01*** | 15 | (0.59) |
| ****01N.02*** | 4 | (0.16) |
| ****01N.83*** | 3 | (0.12) |
| ***RHD(A237D)*** | 3 | (0.12) |
| ****01EL.44*** | 2 | (0.08) |
| ***RHD(c.971delA)[2]*** | 2 | (0.08) |
| ***RHD-CE(3-7)-D-CE(9)-D[2]*** | 2 | (0.08) |
| ***RHDex(4-6)del[2]*** | 2 | (0.08) |
| ****01N.05*** | 1 | (0.04) |
| ****01N.16*** | 1 | (0.04) |
| ****01N.61*** | 1 | (0.04) |
| ****06.02*** | 1 | (0.04) |
| ****15*** | 1 | (0.04) |
| ***RHD(c.52delC)[2]*** | 1 | (0.04) |
| **Total** | 2,540 | (100.00) |

[1]In accordance with the Human RhesusBase[23]. [2]Novel alleles.

**Table II** - *RHD genotype in 1,270 serologically D-negative Thai blood donor samples and associated C/E antigen expression status*

| Allele 1 | Allele 2 | C/E– | | C/E+ | | Total | |
|---|---|---|---|---|---|---|---|
| | | N. | (%) | N. | (%) | N. | (%) |
| **01N.01* | **01N.01* | 670 | (52.76) | 293 | (23.07) | 963 | (75.83) |
| **01EL.01* | **01N.01* | 1 | (0.08) | 162 | (12.76) | 163 | (12.84) |
| **01N.03* | **01N.01* | - | - | 72 | (5.67) | 72 | (5.67) |
| **D-CE(3-10)* | **01N.01* | 1 | (0.08) | 13 | (1.02) | 14 | (1.10) |
| **01* | **01N.01* | - | - | 10 | (0.79) | 10 | (0.79) |
| **01EL.01* | **01EL.01* | - | - | 9 | (0.71) | 9 | (0.71) |
| **01EL.01* | **01N.03* | - | - | 8 | (0.63) | 8 | (0.63) |
| **01N.03* | **01N.03* | - | - | 3 | (0.24) | 3 | (0.24) |
| **01N.83* | **01N.01* | - | - | 3 | (0.24) | 3 | (0.24) |
| **01* | **01EL.01* | - | - | 3 | (0.24) | 3 | (0.24) |
| *RHD(A237D)* | **01N.01* | - | - | 2 | (0.16) | 2 | (0.16) |
| **01N.02* | **01N.01* | - | - | 2 | (0.16) | 2 | (0.16) |
| **01EL.44* | **01N.01* | - | - | 2 | (0.16) | 2 | (0.16) |
| *RHDex(4-6)del*[1] | **01N.01* | - | - | 2 | (0.16) | 2 | (0.16) |
| *RHD(c.971delA)*[1] | **01N.01* | - | - | 2 | (0.16) | 2 | (0.16) |
| **D-CE(3-10)* | **01N.03* | - | - | 1 | (0.08) | 1 | (0.08) |
| **01N.02* | **01N.02* | - | - | 1 | (0.08) | 1 | (0.08) |
| **01N.05* | **01N.01* | - | - | 1 | (0.08) | 1 | (0.08) |
| **01N.16* | **01N.01* | - | - | 1 | (0.08) | 1 | (0.08) |
| **01N.61* | **01N.01* | - | - | 1 | (0.08) | 1 | (0.08) |
| *RHD(c.52delC)*[1] | **01N.01* | - | - | 1 | (0.08) | 1 | (0.08) |
| *RHD-CE(3-7)-D-CE(9)-D*[1] | **01N.01* | - | - | 1 | (0.08) | 1 | (0.08) |
| **01EL.01* | *RHD-CE(3-7)-D-CE(9)-D*[1] | - | - | 1 | (0.08) | 1 | (0.08) |
| *RHD(A237D)* | **01N.03* | - | - | 1 | (0.08) | 1 | (0.08) |
| **06.02* | **01N.01* | - | - | 1 | (0.08) | 1 | (0.08) |
| **15* | **01N.01* | - | - | 1 | (0.08) | 1 | (0.08) |
| **01* | **01* | - | - | 1 | (0.08) | 1 | (0.08) |
| | | 672 | (52.91) | 598 | (47.09) | 1,270 | (100.00) |

[1]Novel alleles.



## Characterization of novel *RHD* alleles

In three donors, *RHD* QMPSF analysis did not show any signal in exons 4, 5, and 6 (*Online Supplementary Content*, **Figure S1A**). This pattern is commonly observed in samples with allele *RHD*06.02* carrying a hybrid *RHD-CE(4-6)-D* gene at the hemizygous state (personal observation). In such a situation, *RHCE* QMPSF typically yields an additional copy of these exons. Here, this latter pattern was confirmed in sample RE055 (i.e. exon 4, 5, and 6 copy number=3), but not in samples NN545 and RS202, in which the copy number remained two (*Online Supplementary Content*, **Figure S1B**). This result suggested that RE055 carries the **06.02* allele, while the other two samples harbor a different allele, possibly involving deletion of *RHD* exons 4, 5, and 6, which has not been reported yet to our knowledge. To address this possibility, we hypothesized that the breakpoints defining a potential deleted region locate within intron 3 and intron 6 at their 5'- and 3'-ends, respectively, and used a series of PCR primer pairs to compare the amplification of *RHD* gene markers in a wild-type control DNA and the samples (**Figure 1A**). The differential patterns obtained by PCR amplification suggested that the potential deletion is located between the exon 3 and intron 6a markers (**Figure 1B**; e3 and i6a, respectively). Subsequent PCR



*Blood Transfus 2023; 21: 209-217* doi: 10.2450/2022.0160-22

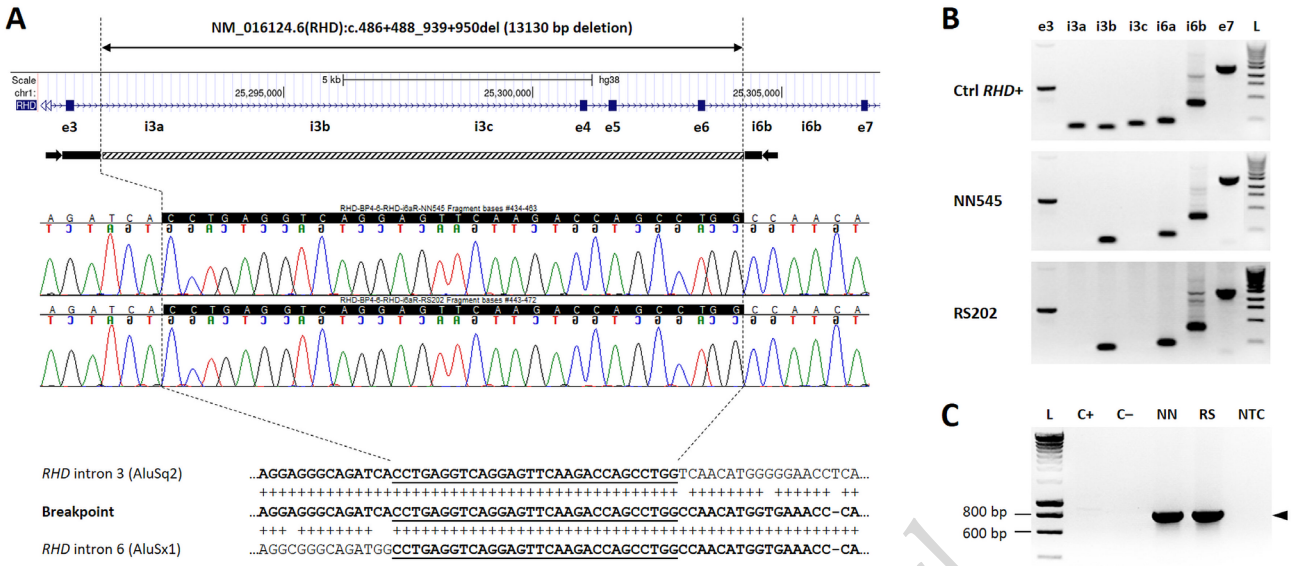


**Figure 1 – Molecular characterization of the novel *RHDex(4-6)del* allele**
(A) Relative positions of the polymerase chain reaction (PCR) markers between *RHD* exons 3 and 7 (top panel); sequencing pattern of the breakpoint in the samples of interest (NN545 and RS202) (middle panel); and sequences in the 30-bp homologous regions (underlined) of introns 3 and 6 resulting in the breakpoint (bottom panel). (B) PCR amplification of specific markers in *RHD* in a wild-type control DNA (Ctrl *RHD*+) and the samples of interest (NN545 and RS202). e3 and e7: *RHD* exons 3 and 7 markers; i3 and i6: *RHD* introns 3 and 6 markers; L: 100 bp DNA ladder. (C) PCR amplification for defining the breakpoint in the novel allele with primers RHD_e3seqF (5'-CAGTCGTCCTGGCTCTCCC-3') and RHD_i6a_R (5'-GCTGGAGTGCCATGGCACG-3') showing a specific PCR product (black arrowhead) in the samples of interest only. C+: wild-type *RHD* control DNA; C–: control DNA defective in the *RHD* gene (homozygous deletion); NN: NN545; RS: RS202; NTC: no template control.

amplification with the respective forward and reverse primers defining the boundaries specifically yielded a single PCR product in the samples, but not in the control DNA samples (**Figure 1C**). Direct sequencing clearly identified the breakpoint, which involves homologous 30-base pair (bp) AluS sequences within intron 3 and intron 6 (**Figure 1A**). The deleted region was thus shown to involve 13,130 base pairs defining the novel *RHDex(4-6)del* allele (NM_016124.6(*RHD*): c.486+489_939+950del) (**Table III**), which is assumed to confer a D– phenotype.

In addition, the *D*-negative *RHD-CE(3-7)-D-CE(9)-D* hybrid allele was found in two samples: NN413 and NN767 (**Table III**). This allele strongly mimics **01N.03*, which is structured as *RHD-CE(3-9)-D*. Actually both alleles share the same open reading frame sequence, because *RHD* and *RHCE* exons 8 are identical, but diverge only by the origin of the exon, which is *RHCE* in **01N.03* and *RHD* in the novel allele as assessed by QMPSF (*data not shown*). Finally, two novel alleles involving a single nucleotide deletion were identified: c.52delC in exon 1, and c.971delA in exon 7, which are both supposed to result in a premature stop codon (**Table III**). Overall, a total of 18 different alleles were identified (**Table I**), including the four novel alleles described above.

**Table III** - *Novel RHD-negative alleles in seven Thai blood donors*

| Novel allele | GenBank Accession Number | Sample ID | RhCE phenotype | *RHD* allele in trans |
|---|---|---|---|---|
| ***RHD(c.52delC)*** | ON229040 | NN444 | ccEe | **01N.01* |
| ***RHD(c.971delA)*** | ON229041 | NN541 | Ccee | **01N.01* |
| | | NN727 | Ccee | **01N.01* |
| ***RHD-CE(3-7)-D-CE(9)-D*** | ON229042 | NN413 | Ccee | **01N.01* |
| | | NN767 | CCee | **01EL.01* |
| ***RHDex(4-6)del*** | ON229043 | NN545 | CcEe | **01N.01* |
| | | RS202 | Ccee | **01N.01* |

### Identification of Asian type *DEL* allele carriers: strategy and molecular assay

Discriminating Asian type DEL individuals, who express the D antigen, from the "true" D– individuals in serologically D– subsets may be of interest in East and Southeast Asia. In a previous study in 1,125 serologically D– Thai blood donors[8], we showed that Asian type *DEL* (**01EL.01*) allele carriers were almost all C/E+ (172/175, 98.3%), while the remaining samples were mostly C/E– (394/950, 41.5%). This observation was confirmed in the current study, in which 183/184 (99.5%) *RHD*01EL.01* allele carriers were C/E+, and, even more specifically, C+, whereas only 415/1,086 (38.2%) were C/E+ among the other samples (**Table II**). On the basis of these data, we consider that screening for the Asian type *DEL* allele in the Thai population may be restricted preferentially to the serologically D–, C+ individuals, Rh C antigen typing being included as an integral part of the whole strategy.

We then thought about designing and implementing a simple molecular assay to identify specifically the c.1227G>A variant, i.e. the molecular SNV involved in the Asian type *DEL* allele. To this aim, we chose to adapt an approach previously used to screen specifically the **01W.01*, **01W.02*, and **01W.03* alleles in the Caucasian population, i.e. the Tm-shift genotyping assay[22]. Briefly, this method is based on real-time PCR amplification using sequence-specific primers of various lengths targeting specifically either the wild-type (c.1227G) or the variant (c.1227A) sequence in *RHD* exon 9. Following melting-curve analysis, samples can be discriminated through their Tm when compared with reference samples. After optimization with a limited number of samples and to

**Table IV** - *RHD alleles with partial deletions (~ 1 kb and longer) and characterized breakpoints*

| Allele designation | Deletion size (bp) | Phenotype | GenBank Accession Number | Reference |
|---|---|---|---|---|
| ***RHD(delEx8)*** | 995 | DEL | N/A | [25] |
| ***RHDex10del type 1*** | 5,405 | Weak D/DEL | JN696682, KX584099 | [26] |
| ***RHD*(Ex3del)602G,667G,819A*** | 10,625 | D– | KY038382 | [27] |
| ***RHDex10del type 2*** | 7,640 | D– | KX584100 | [28] |
| ***RHDex1del type 1*** | 18,450 | DEL | KX584097 | [28] |
| ***RHDex9del*** | 1,012 | DEL | KX793704 | [29] |
| ***RHD*Ex(1-3)del*** | 34,460 | D– | MT231528 | [30] |
| ***RHD*Ex(1-5)del*** | 36,321 | D– | MN783009 | [30] |
| ***RHDex(4-6)del*** | 13,130 | D– | ON229043 | This study |



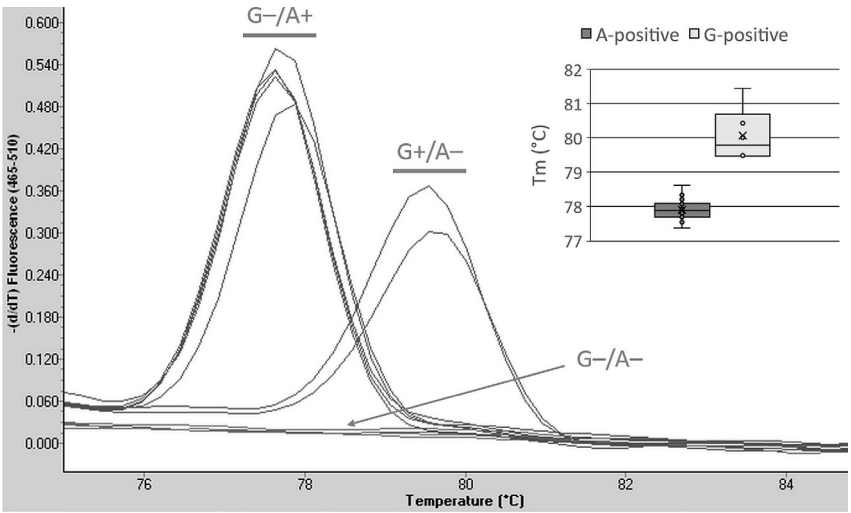


**Figure 2 – Representative profiles of melting-curve analysis for identifying the c.1227G>A single nucleotide variant defining the Asian type *DEL* allele by the Tm-shift genotyping assay.** The embedded graph shows Tm distribution in seven and 62 samples positive for the c.1227G (G-positive; Tm [°C] = 80.06 ± 0.77) and c.1227A (A-positive; Tm [°C] = 77.90°C ± 0.26) specific amplifications, respectively. G–/A+: samples carrying c.1227A only in *RHD* exon 9; G+/A–: samples carrying c.1227G only in *RHD* exon 9; G–/A–: samples negative for both c.1227G and c.1227A.



*Blood Transfus 2023; 21: 209-217* doi: 10.2450/2022.0160-22

test our settings in diagnostics-compatible conditions, C+ samples were selected from the Northeastern and Southern subsets, accounting for a total of 231 samples (*data not shown*). Samples carrying a G and/or an A at position c.1227 in *RHD* exon 9 could be distinguished directly by their Tm (t-test, p-value $<10^{-3}$) (**Figure 2**). More specifically, the Asian type *DEL* allele was identified in 62/231 samples, as expected, with 100% specificity and sensitivity. Overall, our newly designed Tm-shift genotyping assay was validated for accurately identifiying the c.1227G>A SNV in the Thai population.

## DISCUSSION

### *RHD* gene variability in serologically D– Thai blood donors

In this study, we further explore the molecular basis of serological D-negativity in blood donors from three different regions of Thailand: Central, Northeastern, and Southern. In terms of the allele frequency, the four most common variant alleles, i.e. **01N.01*, **01EL.01*, **01N.03* and *RHD-CE(3-10)*, account for ~98.46% of the total *RHD* alleles, which is in agreement with our previous study at the nation-wide level (98.40%)[8], the remaining ~1.5% being shared in 14 other alleles (**Table I**). Data about *RHD* gene polymorphism are thus definitely strengthened in Thailand. It will be very informative to investigate other countries in Southeast Asia at the same level in order to gain insights into *RHD* molecular epidemiology in the area.

In our previous study[8] and this present study in the Thai population, the *RHD* gene has been genotyped in a total of 2,395 serologically D– Thai blood donors. Among these, nine novel alleles were found in 15 donors, i.e. one novel *RHD*-negative allele in every 266 serologically D– Thai individuals. Interestingly, in this study, an allele with a partial deletion encompassing exons 4, 5, and 6 was identified, namely *RHDex(4-6)del* (**Figure 1**). Such mutational events have been rarely reported in *RHD*. Indeed, to our knowledge, this is only the ninth allele described with a ~1 kb or larger deletion (**Table IV**). It is also important to highlight the fact that such an allele would have been misgenotyped if no quantitative method had been used. This further reinforces the statement that, in the context of *RH* molecular genetics, it is important to include (semi)quantitative methods, such as a multiplex ligation-dendent probe amplification assay[31] and the QMPSF[21], in the whole *RH* genotyping strategy, and that these methods play an important role in determining *RHD* zygosity and characterizing alleles involving copy number variations, including gene rearrangements, deletions and duplications[21,32-36]. Overall, the present study definitely extends the molecular knowledge of the *RHD* gene polymorphism.

### Screening for Asian type *DEL* carriers in the serologically D– Thai population

The prevalence of D– individuals has long been known to be <1% in East and Southeast Asia[1-4], resulting in a shortage of D– RBC units and the need for strict control of provision of such units. In this subset in Thailand, we found that ~15% carry the c.1227G>A variant. It is critical to mention that the other exons were not analyzed in this c.1227A-positive subset. Theoretically, an additional variant elsewhere in the gene may result in a different phenotype, e.g. D-negative if carrying a premature stop codon, but this is unlikely. Indeed, so far, to our knowledge, only two alleles carrying the c.1227G>A SNV (i.e. *RHD*01EL.01* and *RHD*01EL.36*, the latter harboring the additional c.1073+152C>A in *RHD* intron 7), both of them resulting in a DEL phenotype, have been officially reported (ISBT Red Cell Immunogenetics and Blood Group Terminology Working Party, RHD Blood Group Allele Table: www.isbtweb.org/resource/004rhd.html), suggesting that other (nonfunctional) alleles on a *RHD*01EL.01* background are very rare, if any. In our previous research in a subset of Thai samples harboring the c.1227G>A SNV, no additional SNV was identified (*personal unpublished data*). On the basis of these observations, we suggest that the samples identified as c.1227A-positive in the current study are very likely to carry the Asian type *DEL* (or *RHD*01EL.01*) allele. These samples are therefore not "true D–" cases, as they are thought to be positive for D antigen expression[14,15]. It is worth mentioning that, because adsorption-elution testing was not carried out, the DEL phenotype was not definitely confirmed in these samples. This phenotype/genotype situation has evident major consequences. From a donor's point of view, mistyping "Asian DEL" RBCs as "D–" RBCs by conventional methods and transfusion of this product have been retrospectively shown to induce alloimmunization in true D– patients[37-41]. From the recipient's point of view, alloimmunization has not been reported in either Asian type DEL pregnant women carrying a D+ fetus or in Asian type DEL

patients transfused with D+ RBC[16,17,19,42,43]. In addition to several lines of biochemical evidence[14,15], these clinical observations strongly suggest that, in the serologically D– subset, Asian type DEL individuals may be preferentially considered as D+, while the molecular mechanisms driving the expression of a complete D antigen remain to be unambiguously determined by functional studies in fresh biological samples to bridge the gap between genotype and phenotype[12]. We, therefore, decided to design a specific strategy with a dedicated flow chart to discriminate specifically Asian type DEL individuals from true D– individuals in Thailand.

Such a strategy has been already implemented in other Asian countries using various PCR-based methods with or without consideration of the Rh CcEe phenotype[44-50]. As indicated above, 359/2,395 (15.0%) serologically D– blood donors were found to carry the *RHD*01EL.01* allele in Thailand. In this subset, 355/359 (98.9%) were not only C/E+, but were actually C+, accounting for ~46% of the total serologically D– Thai blood donors. Overall, the likelihood of finding Asian type *DEL* allele carriers is 32.3% (355/1,102) and 0.3% (4/1,293) in C+ and C–, respectively. Thus, focusing molecular screening on the former subset appears to be a reasonable option for optimizing the cost and probability of identifying the Asian type *DEL* allele, and will be definitely the first stage of selection once serological D– samples are identified.

Next, based on our previous experience in SNV genotyping, we chose to adapt the simple and cost-effective Tm-shift assay approach to identify the c.1227G>A SNV[22]. Our specific assay proved to be accurate to this aim with 100% concordance with the sequencing data. This molecular test will thus be the next stage of selection.

## CONCLUSIONS

Our extensive phenotypic and molecular studies in serologically D– blood donors in the Thai population formed the bases of the design of a specific strategy to distinguish between Asian type DEL and true D– individuals. It will be the role of National Authorities to define precisely the criteria for investigating the c.1227G>A SNV in serologically D– individuals, such as all donors, pregnant women and/or women of childbearing age, patients with a chronic disease, etc. Furthermore, large-scale epidemiological studies in East and Southeast Asia, combined with functional analyses, are now definitely required to address the critical question of the status of Asian type DEL individuals regarding D antigen expression.

## ACKNOWLEDGMENTS

We would like to thank National Blood Center and six Regional Blood Center including KhonKaen, Ubon Ratchathani, Nakhon Ratchasima, Nakhon Si Thammarat, Songkhla, and Phuket Province for providing blood samples, as well as the Reference Laboratory Excellence Center for providing resources. We also express our sincere thanks to Ms. Sineenart Oota (Assistant Director of NBC) and Ms. Supattra Mitundee for supporting the study.

## AUTHORSHIP CONTRIBUTIONS

PN, PK, DC and YF designed the study. JT performed serological and molecular tests. CB performed the molecular characterization of the novel alleles. PN, JT, CF and YF analyzed the results and evaluated the data. PN, JT and YF wrote the paper. CB, PK, DC and CF reviewed and approved the paper.

## FUNDING AND RESOURCES

This project was supported financially by Campus France (Partenariat Hubert Curien [PHC] SIAM, project n°42864RD); the Ministry of Higher Education, Science, Research and Innovation (Franco-Thai Mobility Programme), Bangkok, Thailand; Mahidol University (Basic Research Fund: fiscal year 2021), Nakhon Pathom, Thailand; the Etablissement Francais du Sang (EFS) – Bretagne, and the Institut National de la Sante et de la Recherche Medicale (Inserm), France.

*The Authors declare that they have no conflicts of interest.*

## REFERENCES

1. Daniels G. Rh and RHAG blood group systems. In: Daniels G, editor. *Human blood groups*. 3rd ed. Chichester: Wiley-Blackwell; 2013. p. 182-258.
2. Dewi I, Dalimoenthe NZ, Tjandrawati A, Suraya N. Proportion of Rhesus blood phenotypes at the blood donor unit in Bandung City. Indones J Clin Pathol Med Lab 2019; 25: 155-160. doi: 10.24293/ijcpml.v25i2.807.
3. Fongsarun J, Nuchprayoon I, Yod-in S, et al. Blood groups in Thai blood donors. Thai J Hematol Transfus Med 2002; 12: 277-286.
4. Musa RH, Ahmed SA, Hashim H, Ayob Y, Asidin NH, Choo PY, et al. Red cell phenotyping of blood from donors at the National blood center of Malaysia. Asian J Transfus Sci 2012; 6: 3-9. doi: 10.4103/0973-6247.95042.
5. Shao CP, Maas JH, Su YQ, Köhler M, Legler TJ. Molecular background of Rh D-positive, D-negative, Del and weak D phenotypes in Chinese. Vox Sang 2002; 83: 156-161. doi: 10.1046/j.1423-0410.2002.00192.x.

6. Srijinda S, Suwanasophon C, Visawapoka U, Pongsavee M. RhC phenotyping, adsorption/elution test, and SSP-PCR: the combined test for D-elute phenotype screening in Thai RhD-negative blood donors. ISRN Hematol 2012; 2012: 358316. doi: 10.5402/2012/358316.
7. Kwon DH, Sandler SG, Flegel WA. DEL phenotype. Immunohematology 2017; 33: 125-132. doi: 10.21307/immunohematology-2019-019.
8. Thongbut J, Laengsri V, Raud, Promwong C, I-Na-Ayudhya C, Férec C, et al. Nation-wide investigation of RHD variants in Thai blood donors: impact for molecular diagnostics. Transfusion 2021; 61: 931-938. doi: 10.1111/trf.16242.
9. Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. BMC Genet 2001; 2: 10. doi: 10.1186/1471-2156-2-10.
10. Shao CP, Xiong W, Zhou YY. Multiple isoforms excluding normal RhD mRNA detected in Rh blood group Del phenotype with RHD 1227A allele. Transfus Apheres Sci 2006; 34: 145-152. doi: 10.1016/j.transci.2005.10.001.
11. Liu HC, Eng HL, Yang YF, Wang YH, Lin KT, Wu HL, et al. Aberrant RNA splicing in RHD 7-9 exons of DEL individuals in Taiwan: a mechanism study. Biochim Biophys Acta 2010; 1800: 565-573. doi: 10.1016/j.bbagen.2010.02.006.
12. Fichou Y, Gehannin P, Corre M, Le Guern A, Le Maréchal C, Le Gac G, et al. Extensive functional analyses of RHD splice site variants: insights into the potential role of splicing in the physiology of Rh. Transfusion 2015; 55: 1432-1443. doi: 10.1111/trf.13083.
13. Chen DP, Sun CF, Ning HC, Wang WT, Tseng CP. Comprehensive analysis of RHD splicing transcripts reveals the molecular basis for the weak anti-D reactivity of Del$_I$-red blood cells. Transfus Med 2016; 26: 123-129. doi: 10.1111/tme.12270.
14. Okubo Y, Yamaguchi H, Tomita T, Nagao N. A D variant, Del? Transfusion 1984; 24: 542. doi: 10.1046/j.1537-2995.1984.24685066827.x.
15. Körmöczi GF, Gassner C, Shao CP, Uchikawa M, Legler TJ. A comprehensive analysis of DEL types: partial DEL individuals are prone to anti-D alloimmunization. Transfusion 2005; 45: 1561-1567. doi: 10.1111/j.1537-2995.2005.00584.x.
16. Shao CP. Transfusion of RhD-positive blood in "Asia type" DEL recipients. N Engl J Med 2010; 362: 472-473. doi: 10.1056/NEJMc0909552.
17. Wang QP, Dong GT, Wang XD, Gu J, Li Z, Sun AY, et al. An investigation of secondary anti-D immunisation among phenotypically RhD-negative individuals in the Chinese population. Blood Transfus 2014; 12: 238-243. doi: 10.2450/2013.0184-12.
18. Wang M, Wang BL, Xu W, Fan DD, Peng ML, Pan J, et al. Anti-D alloimmunisation in pregnant women with DEL phenotype in China. Transfus Med 2015; 25: 163-169. doi: 10.1111/tme.12211.
19. Xu W, Zhu M, Wang BL, Su H, Wang M. Prospective evaluation of a transfusion policy of RhD-positive red blood cells into DEL patients in China. Transfus Med Hemother 2015; 42: 15-21. doi: 10.1159/000370217.
20. Thongbut J, Raud L, Férec C, Promwong C, Nuchnoi P, Fichou Y. Comprehensive molecular analysis of serologically D-negative and weak/partial D phenotype in Thai blood donors. Transfus Med Hemother 2020; 47: 54-60. doi: 10.1159/000499087.
21. Fichou Y, Le Maréchal C, Bryckaert L, Dupont I, Jamet D, Chen JM, et al. A convenient qualitative and quantitative method to investigate RHD-RHCE hybrid genes. Transfusion 2013; 53(Suppl 2): 2974-2982. doi: 10.1111/trf.12179.
22. Fichou Y, Le Maréchal C, Jamet D, Bryckaert L, Ka C, Audrézet MP, et al. Establishment of a medium-throughput approach for the genotyping of RHD variants and report of nine novel rare alleles. Transfusion 2013; 53: 1821-1828. doi: 10.1111/trf.12009.
23. Wagner FF, Flegel WA. The Rhesus site. Transfus Med Hemother 2014; 5: 357-363. doi: 10.1159/000366176.
24. Fichou Y, Parchure D, Gogri H, Gopalkrishnan V, Le Maréchal C, Chen JM, et al. Molecular basis of weak D expression in the Indian population and report of a novel, predominant variant RHD allele. Transfusion 2018; 58: 1540-1549. doi: 10.1111/trf.14552.
25. Richard M, Perreault J, Constanzo-Yanez J, Khalifé S, St-Louis M. A new DEL variant caused by exon 8 deletion. Transfusion 2007; 47: 852-857. doi: 10.1111/j.1537-2995.2007.01199.x.
26. Fichou Y, Chen JM, Le Maréchal C, Jamet D, Chuteau C, Durousseau C, et al. Weak D caused by a founder deletion in the RHD gene. Transfusion 2012; 52: 2348-2355. doi: 10.1111/j.1537-2995.2012.03606.x.
27. Hyland CA, Millard GM, O'Brien H, Schoeman EM, Lopez GH, McGowan EC, et al. Non-invasive fetal RHD genotyping for RhD negative women stratified into RHD gene deletion or variant groups: comparative accuracy using two blood collection tube types. Pathology 2017; 49: 757-764. doi: 10.1016/j.pathol.2017.08.010.
28. Srivastava K, Stiles DA, Wagner FF, Flegel WA. Two large deletions extending beyond either end of the RHD gene and their red cell phenotypes. J Hum Genet 2018; 63: 27-35. doi: 10.1038/s10038-017-0345-3.
29. Lopez GH, Turner RM, McGowan EC, Schoeman EM, Scott SA, O'Brien H, et al. A DEL phenotype attributed to RHD exon 9 sequence deletion: splipped-strand mispairing and blood group polymorphisms. Transfusion 2018; 58: 685-691. doi: 10.1111/trf.14439.
30. Matteocci A, Monge-Ruiz J, Stef M, Apraiz I, Herrera-Del-Val L, Mancuso T, et al. Two new RHD alleles with deletions spanning multiple exons. Transfusion 2021; 61: 682-686. doi: 10.1111/trf.16199.
31. Haer-Wigman L, Veldhuisen B, Jonkers R, Loden M, Madgett TE, Avent ND, et al. RHD and RHCE variant and zygosity genotyping via multiplex ligation-dependent probe amplification. Transfusion 2013; 53: 1559-1574. doi: 10.1111/j.1537-2995.2012.03919.x.
32. Ji YL, Luo H, Wen JZ, Haer-Wigman L, Veldhuisen B, Wei L, et al. *RHD* genotype and zygosity analysis in the Chinese Southern Han D+, D– and D variant donors using the multiplex ligation-dependent probe amplification assay. Vox Sang 2017; 112: 660-670. doi: 10.1111/vox.12554.
33. Kulkarni SS, Gogri H, Parchure D, Mishra G, Ghosh K, Rajadhyaksha S, et al. RHD-positive alleles among D– C/E+ individuals from India. Transfus Med Hemother 2018; 45: 173-177. doi: 10.1159/000479239.
34. El Housse H, El Wafi M, Ouabdelmoumene Z, Zarati F, Alid R, Nourichafi N, et al. Comprehensive phenotypic and molecular investigation of RhD and RhCE variants in Moroccan blood donors. Blood Transfus 2019; 17: 151-156. doi: 10.2450/2018.0153-18.
35. Kulkarni SS, Mishra G, Maru H, Parchure D, Gupta D, Bajaj AK, et al. Molecular characterization of rare D--/D-- variants in individuals of Indian origin. Blood Transfus 2022; 20: 59-65. doi: 10.2450/2020.0183-20.
36. Vendrame TAP, Arnoni CP, Latini FRM, Pereira Cortez AJ, Bénech C, Fichou Y, et al. From the investigation of RHD-CE hybrid genes to the recognition of RHCE variants and RHD zygosity – Expanding the analysis by QMPSF in Brazilian donors and in patients with sickle cell disease. Blood Transfus 2022; doi: 10.2450/2022.0028-22. [Online ahead of print.]
37. Yasuda H, Ohto H, Sakuma S, Ishikawa Y. Secondary anti-D immunization by Del red blood cells. Transfusion 2005; 45: 1581-1584. doi: 10.1111/j.1537-2995.2005.00579.x.
38. Kim KH, Kim, KE, Woo KS, Han JY, Kim JM, Park KU. Primary anti-D immunization by DEL red blood cells. Kor J Lab Med 2009; 29: 361-365. doi: 10.3343/kjlm.2009.29.4.361.
39. Shao CP, Wang BY, Ye SH, Zhang WL, Xu H, Zhuang NB, et al. DEL RBC transfusion should be avoided in particular blood recipient in East Asia due to allosensitization and ineffectiveness. J Zhejiang Univ Sci B 2012; 13: 913-918. doi: 10.1631/jzus.B1100348.
40. Yang HS, Lee MY, Park TS, Cho SY, Lee HJ, Lim G, et al. Primary anti-D alloimmunization induced by "Asian type" RHD (c.1227G>A) DEL red cell transfusion. Ann Lab Med 2015; 35: 554-556. doi: 10.3343/alm.2015.35.5.554.
41. Wen J, Wu Y, Wu Y, Zhong C, Jia S, Wei L, et al. Secondary alloanti-D immunization post transfusion of "Asia type" DEL red blood cells. Transfus Apheres Sci 2022; 61: 103458. doi: 10.1016/j.transci.2022.103458.
42. Park JY, Cho D, Choi HW, Jeon MJ, Park MS, Song JW, et al. A patient with RhDel (1227G>A) failed to produce detectable anti-D after transfusion of RhD positive red blood cells. Kor J Blood Transfus 2006; 17: 153-158. doi
43. Choi S, Chun S, Seo JY, Yang JH, Cho D. Planned transfusion of D-positive blood components in an Asia type DEL patient: proposed modification of the Korean National Guidelines for Blood Transfusion. Ann Lab Med 2019; 39: 102-104. doi: 10.3343/alm.2019.39.1.102.
44. Kim JY, Kim SY, Kim CA, Yon GS, Park SS. Molecular characterization of D– Korean persons: development of a diagnostic strategy. Transfusion 2005; 45: 345-352. doi: 10.1111/j.1537-2995.2005.04311.x.
45. Luettringhaus TA, Cho D, Ryang DW, Flegel WA. An easy RHD genotyping strategy for D– East Asian persons applied to Korean blood donors. Transfusion 2006; 46: 2128-2137. doi: 10.1111/j.1537-2995.2006.01042.x.
46. Seo MH, Won EJ, Hong YJ, Chun S, Kwon JR, Choi YS, et al. An effective diagnostic strategy for accurate detection of RhD variants including Asian DEL type in apparently RhD-negative blood donors in Korea. Vox Sang 2016; 111: 425-430. doi: 10.1111/vox.12450.
47. Kim TY, Hong YJ, Kim MJ, Kim H, Kim TS, Park JS, et al. Recommendations regarding practical DEL typing strategies for serologically D-negative Asian donors. Transfus Med Hemother 2020; 47: 88-93. doi: 10.1159/000500098.
48. Chun S, Kim H, Yun JW, Yu HB, Seo JY, Cho D. *RHD* genotyping is recommended for all patients with serological weak-D phenotypes in Asian populations – cases with coexistence of weak-D and Asia type DEL alleles results in complete expression of D-antigen. Transfus Apheres Sci 2020; 59: 102807. doi: 10.1016/j.transci.2020.102807.
49. Ito S, Ohto H, Ogiyama Y, Irino M, Omokawa S, Shibasaki I, et al. A practical and effective strategy in East Asia to prevent anti-D alloimmunization in patients by C/c phenotyping of serologic RhD-negative blood donors. eJHaem 2021; 2: 750-756. doi: 10.1002/jha2.292.
50. Yin Q, Flegel WA. DEL in China: the D antigen among serologic RhD-negative individuals. J Transl Med 2021; 19: 439. doi: 10.1186/s12967-021-03116-6.