

## Genotyping-based prediction of Diego blood group phenotypes: Implications for Thai blood donors

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### ABSTRACT

**Background:** The Di<sup>a</sup> and Di<sup>b</sup> antigens of the Diego (DI) blood group system can cause alloimmunization following transfusion or pregnancy. Although the Di(a-b+) phenotype predominates, the Di(a+) antigen is relatively common in Asian populations. Due to serological limitations, particularly the lack of anti-Di<sup>b</sup>, genotyping is increasingly used to predict DI phenotypes and facilitate identifying antigen-negative blood donors for patients with anti-Di<sup>a</sup> or anti-Di<sup>b</sup>.

**Objectives:** The study aimed to determine the frequencies of *DI*\*A and *D*\*B alleles to predict DI phenotypes and establish an inventory of Thai blood donors with the rare Di(a+b-) phenotype.

**Materials and methods:** A total of 3,500 blood samples from unrelated healthy Thai blood donors were included. All samples were tested with anti-Di<sup>a</sup> using the conventional tube technique and genotyped for *DI*\*A and *DI*\*B alleles by in-house polymerase chain reaction with sequence-specific primer technique. The DI allele frequencies were calculated to estimate the risk of alloimmunization.

**Results:** The DI genotyping and phenotyping results showed 100% concordance. Among 3,500 Central Thai blood donors, predicted phenotypes were ranked as Di(a-b+), 3,407 (97.34%), followed by Di(a+b+), 92 (2.63%), and Di(a+b-), 1 (0.03%). The *DI*\*A and *DI*\*B allele frequencies were 0.013 and 0.987. The *DI*\*A and *DI*\*B frequencies are consistent with those observed in Central and Northern Thai populations and in other Southeast Asian populations, but differ significantly from those observed in Southern Thai, Japanese, Chinese, and Korean populations ( $p < 0.001$ ). Notably, one donor with the rare Di(a+b-) phenotype was observed in this study; this likely reflects the greater likelihood of detecting rare phenotypes when screening a much larger sample size than in related Thai studies.

**Conclusion:** The implementation of the PCR-SSP technique plays a crucial role in identifying DI blood group antigens, particularly in situations where specific antisera are unavailable. This approach also contributes to establishing an inventory of donors with rare blood phenotypes, thereby enhancing transfusion safety.

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### Introduction

The Diego (DI) blood group system is clinically important in transfusion practice. The International Society of Blood Transfusion (ISBT) has assigned 23 antigens located in the integral band 3 glycoprotein of the red blood cell (RBC) membrane.<sup>1</sup> Two closely related antigen pairs of the DI system are Di<sup>a</sup>/Di<sup>b</sup> and Wr<sup>a</sup>/Wr<sup>b</sup>

Of these,  $\text{Di}^b$  and  $\text{Wr}^b$  are high-prevalence antigens, while  $\text{Di}^a$  and  $\text{Wr}^a$  are low-prevalence antigens.<sup>2-4</sup> Importantly, the  $\text{Di(a+)}$  antigen is relatively common in Asian populations. Its frequency ranges from 2 to 12% in Eastern Asian populations.<sup>4</sup> Regarding the data on three DI phenotypes among populations,  $\text{Di(a-b+)}$  is the most frequent. This is followed by  $\text{Di(a+b+)}$ , while  $\text{Di(a+b-)}$  is a rare phenotype. In Thai donor populations, the predicted  $\text{Di(a+)}$  antigen frequency of only  $\text{Di(a+b+)}$  phenotype was 3.70% among Central and Northern Thais and 0.94% among Southern Thais.<sup>5-8</sup> Although anti- $\text{Di}^a$  and anti- $\text{Di}^b$  are involved in mild to severe cases of hemolytic disease of the fetus and newborn (HDFN) and hemolytic transfusion reactions (HTRs),<sup>2,3</sup> anti- $\text{Di}^a$  is more frequently identified among pregnant women and patients with repeated transfusions. This is due to the high prevalence of the  $\text{Di(a+b+)}$  phenotype across various populations.<sup>2-4,9-11</sup>

In general, to determine  $\text{Di(a+)}$  and  $\text{Di(a-)}$  phenotypes, RBCs are tested with anti- $\text{Di}^a$ , using the conventional tube technique (CTT) or column agglutination technique (CAT). The phenotypic differentiation between  $\text{Di(a+b+)}$  and  $\text{Di(a+b-)}$  is inconclusive with CTT or CAT due to the lack of an anti- $\text{Di}^b$ . It is known that the  $\text{Di}^a$  and  $\text{Di}^b$  antigens are encoded by the  $\text{DI}^*A$  and  $\text{DI}^*B$  alleles, respectively, which arise from a single nucleotide variant (SNV), c.2561C>T in exon 19 of the human erythrocyte membrane anion-transporter gene (*SLC4A1*). This polymorphism results in a p.(Pro854Leu) amino acid substitution that distinguishes the  $\text{Di}^a$  and  $\text{Di}^b$  antigens. In recent years, molecular blood group typing has become an important tool in transfusion medicine. Related studies from our group in Thailand demonstrated that DI genotyping accurately predicts  $\text{Di}^a$  and  $\text{Di}^b$  antigens in the absence of commercially available anti- $\text{Di}^b$  reagent, enabling discrimination between the  $\text{Di(a+b+)}$  and rare  $\text{Di(a+b-)}$  phenotypes that cannot be distinguished by anti- $\text{Di}^a$  serology alone. Hence, different genotyping techniques have been used to determine  $\text{DI}^*A$  and  $\text{DI}^*B$  alleles to predict accurate DI phenotypes among donors and patients, e.g., PCR with sequence-specific primer (PCR-SSP),<sup>5-8,12</sup> real-time PCR,<sup>13</sup> high-resolution melting curve analysis (HRM),<sup>13,14</sup> microarrays<sup>10,11</sup> and DNA sequencing.<sup>5-8,11-14</sup>

In one patient with  $\text{Di(a+b-)}$  phenotypes receiving  $\text{Di(a+)}$  from either  $\text{Di(a+b+)}$  or  $\text{Di(a+b-)}$  blood donors, anti- $\text{Di}^a$  was produced, making a compatible blood transfusion easy. In contrast, for  $\text{Di(a+b-)}$  patients who have developed anti- $\text{Di}^b$ , finding compatible antigen-negative blood donors can be extremely difficult, potentially leading to significant delays in transfusion support. Hence, correctly detecting DI blood group antigens, particularly among patients receiving chronic transfusion therapy, is essential to prevent further problems of incompatible transfusions and to ensure the appropriate use of blood units with low-prevalence phenotypes; thereby, promoting safe and effective transfusion support.

In Thailand, the availability of blood donors with the rare  $\text{Di(a+b-)}$  phenotype remains unclear because related studies were limited by the unavailability of anti- $\text{Di}^b$  reagent and relatively small sample sizes. Expanding the study population enables a more precise estimation of DI allele and phenotype frequencies and increases the likelihood of identifying individuals with the rare  $\text{Di(a+b-)}$  phenotype. Establishing an inventory of these rare donors would facilitate timely provision of compatible blood for patients with anti- $\text{Di}^b$ ; thereby, improving transfusion support for this clinically challenging patient group.<sup>5</sup>

Therefore, this study aimed to determine the frequencies of the  $\text{DI}^*A$  and  $\text{DI}^*B$  alleles to predict DI phenotypes and establish an inventory of Thai blood donors possessing the rare  $\text{Di(a+b-)}$  phenotype.

## Materials and methods

### Sample collection

Leftover EDTA-anticoagulated blood samples were collected from 3,500 unrelated, healthy Thai donors at the Blood Bank, Thammasat University Hospital, Pathum Thani, Thailand. Written informed consent was obtained from each subject. The sample size was determined using a single-population proportion formula based on the prevalence of the  $\text{Di(a+b-)}$  phenotype among Thais (0.6%).<sup>15</sup> Assuming a 95% confidence level and a margin of error of 0.08%, the minimum required sample size was estimated to be 3,500 blood donors. Inclusion criteria for each subject include Thai ethnicity and meeting the donor selection criteria according to the Guidelines of the National Blood Centre, Thai Red Cross Society.<sup>16</sup> Donors with positive infectious marker testing were excluded.

### DNA standards

Known DNA samples comprising 1  $\text{Di(a+b-)}$ , 5  $\text{Di(a+b+)}$  and 1  $\text{Di(a+b-)}$  phenotypes confirmed by DNA sequencing in an earlier study,<sup>5</sup> were used as controls.

### ABO blood group and $\text{Di}^a$ antigen testing using serological techniques

All blood samples were tested for ABO cell and serum grouping by CAT using an ORTHO VISION Analyzer (Ortho-Clinical Diagnostics, Raritan, NJ, USA).  $\text{Di}^a$  antigen detection was performed using the CTT. Briefly, 1 drop of monoclonal human anti- $\text{Di}^a$  (National Blood Centre, Thai Red Cross Society, Bangkok, Thailand) was added, followed by 1 drop of a 3-5% red cell suspension in 0.9% normal saline. The test tube was mixed and incubated at room temperature for 10 minutes. After centrifugation, the reactions were examined macroscopically, and agglutination strength was graded as 4+, 3+, 2+, 1+, weakly positive (w+) or negative (0). Negative,  $\text{Di(a+b+)}$  and positive,  $\text{Di(a+b+)}$  control cells were also tested in parallel.

### DI\*A and DI\*B allele genotyping

Genomic DNA was extracted from peripheral blood samples using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany), according to manufacturer instructions. DNA samples were further stored at -20 °C until use for genotyping. Genotyping of DI\*A and DI\*B alleles was performed using an in-house PCR-SSP technique, with modifications.<sup>5</sup> Primers and amplification conditions were identical to a related study.<sup>5</sup> Briefly, 1 µL of genomic DNA (50 ng/µL) was amplified in a total volume of 10 µL using 5 µM of DI-AB-F forward primer 5'-GGTGTGATAGGCACTGACCC-3', 1 µL and 5 µM DI-A-R reverse primer 5'-GGGCCAGGG AGGCCA-3', 1 µL for DI\*A allele detection and 5 µM of DI-AB-F forward primer, 1 µL for DI-B-R reverse primers 5'-GGGCCAGGGAGGCCG-3', and 1 µL for DI\*B allele detection. Co-amplification of the HGH gene using 6 µM HGH forward primer 5'-TGCCTTCCCAA CCATTCCCTTA-3', 1 µL, and 6 µM HGH reverse primer 5'-CCACTCACGGATTCTGTTGTGTTTC-3', 1 µL was run as the internal control and 5 µL of 2X PCR reaction mixture (Green Hot Start, Biotechrabbit, Berlin, Germany) in the thermal cycler (Applied Biosystems™ VeritiPro™).

PCR was performed with an initial denaturation at 95 °C for 30 seconds, followed by 10 cycles of 95 °C for 30 seconds and 69 °C for 10 seconds, then 20 cycles of 95 °C for 10 seconds, 62 °C for 50 seconds, and 72 °C for 30 seconds, with a final extension at 72 °C for 5 minutes and a 4 °C hold. PCR products were separated using electrophoresis on a 1.5% agarose gel in 1x TBE buffer at 100 V and visualized under a blue-light transilluminator. The expected amplicon size was 130 bp for both the DI\*A and DI\*B alleles, with a 434-bp HGH gene fragment serving as the internal control.

Known DNA samples of Di(a+b-), Di(a+b+) and Di(a-b+) phenotypes were used as controls. A total of 3,500 DNA samples of Thai blood donors were tested for the detection of the DI\*A and DI\*B alleles using PCR-SSP.

### DNA sequencing

To confirm the PCR-SSP results, DNA samples from 50 genotyped blood donors (1 DI\*A/A, 4 DI\*A/B, and 45 DI\*B/B) were sequenced using U2Bio DNA Sequencing Services (Bangkok, Thailand). A 598-bp fragment encompassing the c.2561C>T SNP was amplified using forward and reverse primers, identical to those used in a related study under the same PCR conditions used for DI genotyping.<sup>5</sup>

### Statistical analysis

Gene frequencies were calculated using the gene counting method. The Chi-square ( $\chi^2$ ) test was used to evaluate whether the observed genotype frequencies agreed with the expected ones under the Hardy-Weinberg equilibrium. The  $\chi^2$  test of homogeneity was

used to determine the difference between Central Thai (this study) and those reported in Thai and other Asian populations,<sup>5-8</sup> including Han Chinese (Shanghai),<sup>17</sup> Chinese (Shen-Zhen),<sup>18</sup> Chinese (Chengdu),<sup>19</sup> Japanese,<sup>10</sup> Korean,<sup>10</sup> Southeast Asian<sup>10</sup> and Filipino,<sup>10</sup> respectively. All statistical analyses were conducted using IBM SPSS Statistics, Version 26.0 (SPSS Inc., Chicago, IL, USA). A  $p=0.05$  or less was considered significant.

In addition, the percentage of predicted Di<sup>a</sup>/Di<sup>b</sup> incompatibilities was calculated using the formula:  $[DI^*A/A (DI^*B/B + DI^*A/B) + DI^*B/B (DI^*A/A + DI^*A/B)]$ ; DI\*A/A, DI\*A/B and DI\*B/B representing the genotype frequencies in each population. The estimated risk of Di<sup>a</sup>/Di<sup>b</sup> alloimmunization was determined by multiplying the probability of being predicted by the Di<sup>a</sup>/Di<sup>b</sup> negative phenotype frequency by the probability of having a predicted Di<sup>a</sup>/Di<sup>b</sup> phenotype frequency,<sup>18</sup> i.e., anti-Di<sup>a</sup> or anti-Di<sup>b</sup>, by considering the Di<sup>a</sup>/Di<sup>b</sup> pair of antigens under four conditions described below:

1. Di<sup>a</sup>/Di<sup>b</sup> donor to Di<sup>b</sup>/Di<sup>b</sup> patient (risk of anti-Di<sup>a</sup> production):  $Di^a/Di^b \times Di^b/Di^b$
2. Di<sup>a</sup>/Di<sup>a</sup> donor to Di<sup>b</sup>/Di<sup>b</sup> patient (risk of anti-Di<sup>a</sup> production):  $Di^a/Di^a \times Di^b/Di^b$
3. Di<sup>b</sup>/Di<sup>b</sup> donor to Di<sup>a</sup>/Di<sup>a</sup> patient (risk of anti-Di<sup>b</sup> production):  $Di^b/Di^b \times Di^a/Di^a$
4. Di<sup>a</sup>/Di<sup>b</sup> donor to Di<sup>a</sup>/Di<sup>a</sup> patient (risk of anti-Di<sup>b</sup> production):  $Di^a/Di^b \times Di^a/Di^a$

Overall, the global risk of alloimmunization associated with Di<sup>a</sup>/Di<sup>b</sup> incompatibility is calculated as the sum of the risks across the four conditions.

## Results

### Participants data

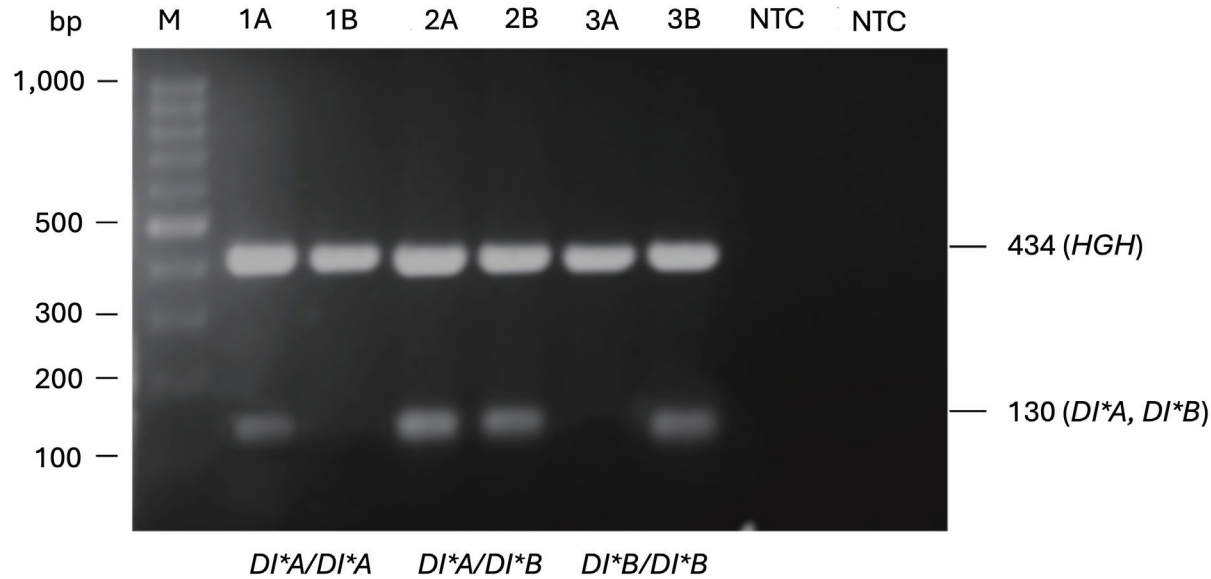
Among 3,500 central Thai blood donors, ages ranged from 18 to 65 years. For the ABO blood group, group O was the most common (1,347, 38.49%) followed by group B (1,118, 31.94%), group A (760, 21.71%), and group AB (275, 7.86%).

### Serological testing for Di<sup>a</sup> antigen

The distribution Di<sup>a</sup> antigen among 3,500 blood donor samples was analyzed. Of these, 93 donors (2.66%) were Di(a+), while 3,407 donors (97.34%) were Di(a-).

### Genotyping results of DI\*A and DI\*B alleles

Genotyping of DI\*A and DI\*B alleles using an in-house PCR-SSP was successfully performed among all participants. Genotypes were determined by the specific product size, as described in the materials and methods section. The genotype results in this study demonstrated that the PCR product sizes corresponded to the three expected DI genotypes DI\*A/A, DI\*A/B, and DI\*B/B, as shown in Figure 1.



**Figure 1.** A representative gel showing Diego blood group genotyping using the PCR-SSP technique. The 434 bp amplification product of the HGH control primer was present in all lanes, confirming successful amplification. Genotypes were determined based on the presence or absence of amplification products specific to alleles of *DI\*A*(A) and *DI\*B* (B). Lanes 1A-1B: *DI\*A/A*, Lanes 2A-2B: *DI\*A/B*, Lanes 3A-3B: *DI\*B/B*, NTC: nontemplate control, Lane M: 100 bp ladder marker (Fermentas, Carlsbad, CA, USA). Arrows indicate the size of *DI* gene fragments (right): *DI\*A* and *DI\*B* (130 bp), and HGH internal control (434 bp).

*DI\*A* and *DI\*B* genotyping results using PCR-SSP among 3,500 donor samples with known Di(a+) and Di(a-) status were used to predict DI phenotypes and agreed with the phenotyping results using serological testing. In addition, the DI genotypes were ranked in order as *DI\*B/B* (3,407/3,500, 97.34%), *DI\*A/B* (92/3,500, 2.63%), and *DI\*A/A* (1/3,500, 0.03%). The

determined *DI\*A* and *DI\*B* genotypes among 3,500 Thai blood donors were consistent with the Hardy-Weinberg equilibrium ( $\chi^2=0.045$ ,  $p=0.830$ ). Allele frequencies of *DI\*A* and *DI\*B* among Central Thai blood donors in this study were 0.013 (94/7,000) and 0.987 (6,906/7,000), respectively (Table 1).

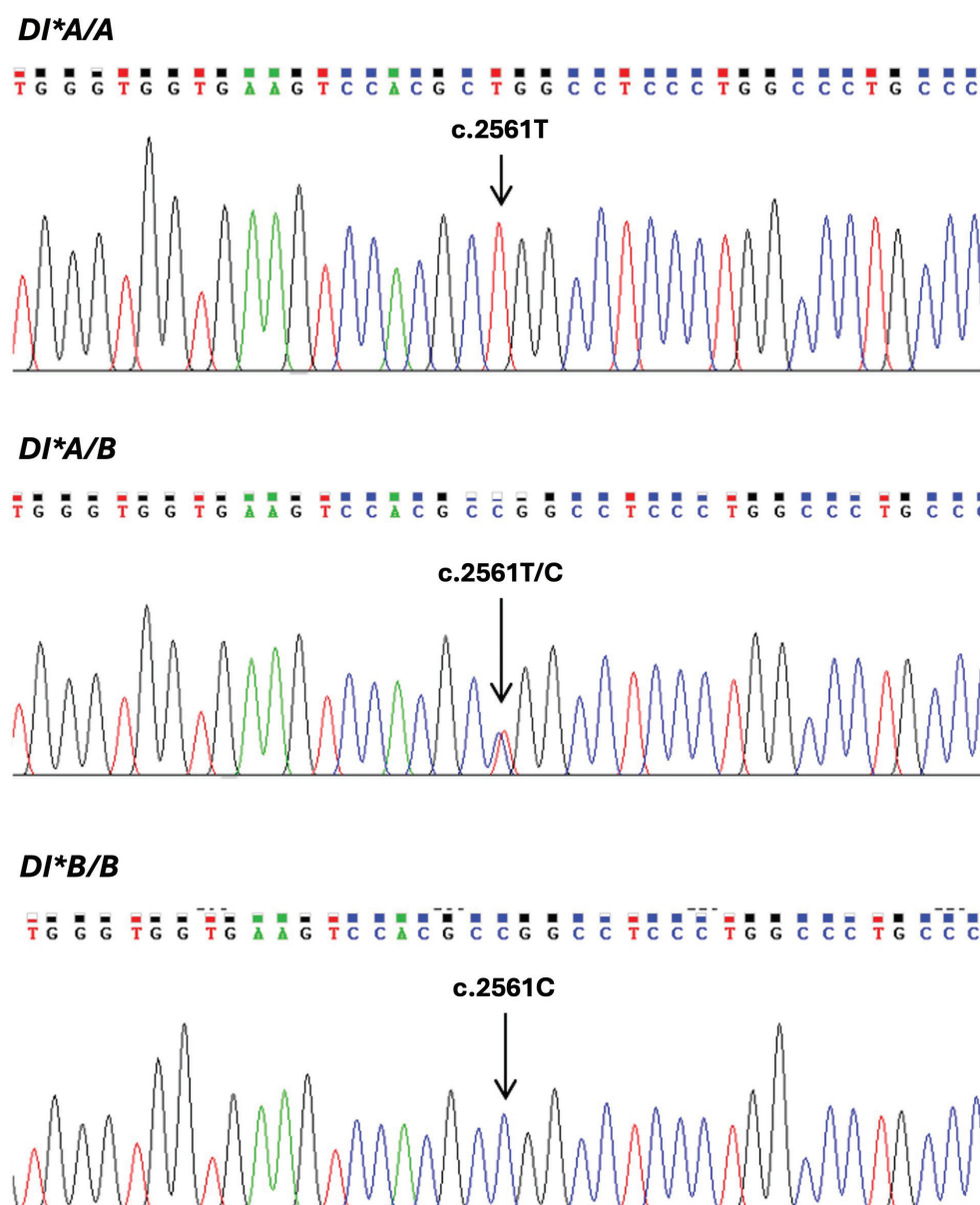
**Table 1.** *DI\*A* and *DI\*B* genotype and allele frequencies in Central Thai donor populations (N=3,500).

| Allele      | Allele frequency | Genotype      | Observed (%) | Expected (HWE) | $\chi^2$ | $p$ (DF=1) |
|-------------|------------------|---------------|--------------|----------------|----------|------------|
| <i>DI*A</i> | 94 (0.013)       | <i>DI*A/A</i> | 1            | 1              | 0.045    | 0.830      |
| <i>DI*B</i> | 6,906 (0.987)    | <i>DI*A/B</i> | 92           | 90             |          |            |
|             |                  | <i>DI*B/B</i> | 3,407        | 3,409          |          |            |

**Note:** DF: Degree of freedom, HWE: Hardy-Weinberg equilibrium.

Moreover, the in-house PCR-SSP assay was validated against serological DI phenotyping, the gold standard for Di<sup>a</sup> antigen determination, and demonstrated complete agreement for all tested samples, yielding 100% sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and

overall accuracy. Furthermore, validation of the assay using fifty randomly selected samples representing the *DI*<sup>a</sup>/*A*, *DI*<sup>a</sup>/*B*, and *DI*<sup>b</sup>/*B* genotypes demonstrated 100% concordance between the PCR-SSP results and DNA sequencing (Figure 2).



**Figure 2.** Electropherograms of the *DI* gene at the *DI*<sup>a</sup> and *DI*<sup>b</sup> polymorphism (SNP c.2561C>T). DNA sequences shown are 1: *DI*<sup>a</sup>/*A* homozygote, 2: *DI*<sup>a</sup>/*B* heterozygote, 3: *DI*<sup>b</sup>/*B* homozygote.

The *DI*\*A and *DI*\*B genotype and allele frequencies among 3,500 Central Thai blood donors, as well as other Thai and Asian populations, are presented in Table 2. No significant difference was observed between Central Thai (this study) and the other three Thai populations, including Central Thai,<sup>5</sup> Northern Thai,<sup>6</sup> Northeastern

Thai,<sup>7</sup> and Southeast Asian<sup>10</sup> populations ( $p>0.05$ ). Significant differences in *DI*\*A and *DI*\*B frequencies were observed when comparing Central Thai blood donors with Southern Thai,<sup>8</sup> Filipino,<sup>10</sup> Han Chinese (Shanghai),<sup>17</sup> Chinese (Shen-Zhen),<sup>18</sup> Chinese (Chengdu),<sup>19</sup> Japanese<sup>10</sup> and Korean<sup>10</sup> populations ( $p<0.05$ ).

**Table 2.** *DI*\*A and *DI*\*B allele frequencies in Asian populations.

| Population                           | Number | Allele frequencies |               | $\chi^2$ | p value |
|--------------------------------------|--------|--------------------|---------------|----------|---------|
|                                      |        | <i>DI</i> *A       | <i>DI</i> *B  |          |         |
| Central Thai*                        | 3,500  | 94 (0.013)         | 6,906 (0.987) | -        | -       |
| Central Thai <sup>5</sup>            | 1,011  | 37 (0.018)         | 1,985 (0.982) | 2.600    | 0.1068  |
| Northern Thai <sup>6</sup>           | 300    | 11 (0.018)         | 589 (0.982)   | 0.976    | 0.3233  |
| Northeastern Thai <sup>7</sup>       | 345    | 4 (0.006)          | 686 (0.994)   | 2.907    | 0.0882  |
| Southern Thai <sup>8</sup>           | 427    | 4 (0.005)          | 850 (0.995)   | 4.724    | <0.05   |
| Filipino <sup>10</sup>               | 1,333  | 21 (0.008)         | 2,645 (0.992) | 5.062    | <0.05   |
| Southeast Asian <sup>10</sup>        | 942    | 28 (0.015)         | 1,856 (0.985) | 0.225    | 0.6351  |
| Han Chinese (Shanghai) <sup>17</sup> | 403    | 36 (0.045)         | 770 (0.955)   | 43.063   | <0.001  |
| Chinese (Shen-Zhen) <sup>18</sup>    | 1,766  | 126 (0.036)        | 3,406 (0.964) | 56.799   | <0.001  |
| Chinese (Chengdu) <sup>19</sup>      | 300    | 36 (0.060)         | 564 (0.940)   | 71.292   | <0.001  |
| Japanese <sup>10</sup>               | 1,022  | 88 (0.043)         | 1,956 (0.957) | 70.410   | <0.001  |
| Korean <sup>10</sup>                 | 1,033  | 120 (0.058)        | 1,946 (0.942) | 138.014  | <0.001  |

**Note:** \*Central Thai in this study compared to other populations.

The *Di*<sup>a</sup>/*Di*<sup>b</sup> incompatibilities were predicted among Central Thais in this study with related reports in Thai populations (Table 3). The *Di*<sup>a</sup>/*Di*<sup>b</sup> incompatibilities among Central Thais were 2.62%, which were closely related to those of Central Thais<sup>5</sup> (3.53%) and Northern Thais<sup>6</sup> (3.54%). The lowest percentage of predicted *Di*<sup>a</sup>/*Di*<sup>b</sup> incompatibilities was observed in Southern Thais<sup>8</sup> (0.93%), followed by Northeastern Thais (1.15%).<sup>7</sup> In addition, the risk of *Di*<sup>a</sup> and *Di*<sup>b</sup> alloimmunization

among different Thai populations was analyzed (Table 3). An important finding of our study was that, according to the *DI* genotype data, the estimated risk of *Di*<sup>a</sup> alloimmunization in Central and Northern Thai populations was about 2.8-3.8-fold higher than in Southern Thai populations (Table 4). Importantly, the estimated risk of *Di*<sup>b</sup> alloimmunization was very low (0.0003) in the Central Thai population, whereas its risk was 0.00 in other Thai populations.

**Table 3.** Estimated risks of *Di*<sup>a</sup> and *Di*<sup>b</sup> incompatibility and alloimmunization in Thai populations.

| Population                     | Number | Predicted <i>Di</i> <sup>a</sup> Phenotype |          | <i>Di</i> <sup>a</sup> incompatibilities (%) | Alloimmunization risks |                        |
|--------------------------------|--------|--------------------------------------------|----------|----------------------------------------------|------------------------|------------------------|
|                                |        | Negative                                   | Positive |                                              | <i>Di</i> <sup>a</sup> | <i>Di</i> <sup>b</sup> |
| Central Thai*                  | 3,500  | 0.973                                      | 0.027    | 2.62                                         | 0.026                  | 0.0003                 |
| Central Thai <sup>5</sup>      | 1,011  | 0.963                                      | 0.037    | 3.53                                         | 0.035                  | 0.0000                 |
| Northern Thai <sup>6</sup>     | 399    | 0.964                                      | 0.036    | 3.54                                         | 0.035                  | 0.0000                 |
| Northeastern Thai <sup>7</sup> | 345    | 0.988                                      | 0.012    | 1.15                                         | 0.012                  | 0.0000                 |
| Southern Thai <sup>8</sup>     | 427    | 0.991                                      | 0.009    | 0.93                                         | 0.009                  | 0.0000                 |

**Note:** \*this study.

**Table 4.** Predicted DI phenotypes according to ABO blood group among 3,500 blood donors.

| DI phenotype | ABO blood group (%)   |                       |                     |                     |
|--------------|-----------------------|-----------------------|---------------------|---------------------|
|              | O                     | B                     | A                   | AB                  |
| Di(a+b-)     | 1 (0.07)              | 0 (0.00)              | 0 (0.00)            | 0 (0.00)            |
| Di(a+b+)     | 32 (2.38)             | 37 (3.31)             | 14 (1.84)           | 9 (3.27)            |
| Di(a-b+)     | 1,314 (97.55)         | 1081 (96.69)          | 746 (98.16)         | 266 (96.73)         |
| <b>Total</b> | <b>1,347 (100.00)</b> | <b>1,118 (100.00)</b> | <b>760 (100.00)</b> | <b>275 (100.00)</b> |

Additionally, the distribution of ABO blood groups and the frequency of Di(a+b+) phenotypes were evaluated among 3,500 blood donors. The highest frequency of Di(a+b+) phenotype was observed in group B (37/1,118, 3.31%), followed by group AB (9/275, 3.27%), group O (33/1,347, 2.45%), and group A (14/760, 1.84%). Although the frequency of the Di(a+b+) phenotype was higher among individuals with blood group B compared with all other blood groups combined (OR=1.41, 95%CI: 0.92-2.16,  $p=0.11$ ), and lower in those with blood group A (OR=0.64, 95%CI: 0.36-1.13,  $p=0.13$ ), these differences were not statistically significant.

## Discussion

Because the Di(b+) antigen is highly prevalent across populations, anti-Di<sup>a</sup> is more frequently detected than anti-Di<sup>b</sup> among pregnant women and multi-transfused patients.<sup>3,4,12</sup> In a study conducted at the National Blood Centre, Thai Red Cross Society (NBC-TRC), only anti-Di<sup>a</sup> was identified in 1,766 multi-transfused Thai patients (1.20%).<sup>21</sup> A severe case of delayed HTRs and a case series of HDFN caused by anti-Di<sup>b</sup> were reported in Japanese populations.<sup>22,23</sup> Recently, a case reported by the NBC-TRC was observed in which a mother with panagglutinating antibodies was ultimately confirmed to have anti-Di<sup>b</sup>. She delivered a newborn with a Di(a+b+) phenotype, without evidence of anemia or jaundice (unpublished data). Hence, anti-Di<sup>b</sup> among Thai patients may be reported as an unidentified antibody because it agglutinates all reagent RBCs in the antibody identification panel, necessitating the use of additional panel cells with the Di(a+b-) phenotype for accurate identification.

Commercial anti-Di<sup>b</sup> reagent is unavailable to distinguish between the Di(a+b+) and Di(a+b-) phenotypes; therefore, genotyping is recommended and has been widely used in population studies.<sup>3,5-8,10-19</sup> Related studies in Thai populations have reported only the Di(a+b+) phenotype among individuals with Di(a+), whereas Di(a+b-) was not observed,<sup>5-8</sup> possibly due to limited sample sizes. Larger studies are required to better assess the risk of alloimmunization and to find matched blood donors for alloimmunized patients. A related study has demonstrated that DI genotyping using the in-house PCR-SSP assay showed complete concordance with serological phenotyping and DNA

sequencing results. Furthermore, this method is simple, convenient, and cost-effective compared with commercially available PCR-based assays.<sup>5</sup>

In this study, we performed DI genotyping using in-house PCR-SSP in 3,500 known Di<sup>a</sup> antigen phenotype samples; the predicted DI phenotypes revealed 100% concordance with the phenotyping results. The validity of in-house PCR-SSP for DI genotyping was consistent with the determined genotypes by DNA sequencing. Importantly, one homozygous *DI*<sup>a</sup>/*A* group O blood donor was found among 3,500 blood donors. The probability of finding an extended-match donor with the Di(a+b-) phenotype for a patient developing anti-Di<sup>b</sup> is 0.03%. Identifying this phenotype should be incorporated in a rare donor registry to enable rapid identification and recall when compatible blood is required. Molecular characterization of rare donors can complement conventional serological screening, particularly where anti-Di<sup>b</sup> reagents are unavailable, and facilitate the provision of compatible RBC units for patients with anti-Di<sup>b</sup>. Therefore, mass donor screening may help expand the rare donor registry and improve the availability of compatible blood for future transfusion support in the Thai population.

A related study conducted by the NBC-TRC reported the frequencies of Di<sup>a</sup> and Di<sup>b</sup> antigens among 5,285 Thai blood donors included in the rare blood type inventory, as well as in reagent screening and panel cells.<sup>24</sup> The results showed that 11 donors exhibited the Di(a+b-) phenotype (0.21%). However, the samples were not randomly selected, so the data could not accurately assess the risk of alloimmunization.

No significant differences were found in *DI*<sup>a</sup>/*A* and *DI*<sup>b</sup>/*B* frequencies between Central Thai (this study) and the other three Thai populations: Central Thai,<sup>5</sup> Northern Thai<sup>5</sup>, and Northeastern Thai.<sup>7</sup> However, significantly lower frequencies were observed only among Southern Thais,<sup>8</sup> resulting in an increased risk of Di<sup>a</sup> incompatibility in Central Thai populations. Similar significant differences in allele frequencies were observed when comparing Central Thai blood donors with Filipino, Chinese, Japanese, and Korean populations ( $p<0.05$ ).<sup>10,17-19</sup>

Furthermore, the prevalence of the Di(a+b+) phenotype among donors was analyzed by ABO blood group. No statistically significant differences were

observed between groups, suggesting no evidence of an association between the Di(a+) phenotype and ABO blood group in this study.

### Limitations

This study encountered several limitations. Firstly, only one donor with the rare Di(a+b-) phenotype was identified, limiting the ability to draw broader conclusions about the prevalence and characteristics. Secondly, this constituted a single-center study including only Central Thai blood donors, which may limit the generalizability of the findings to other regions of the country. Thirdly, DNA sequencing was performed to validate the PCR-SSP results in only a subset of samples rather than the entire study population. Finally, the study did not evaluate clinical outcomes such as the impact of DI genotyping on transfusion management or patient outcomes. Therefore, multicenter studies involving larger and more geographically diverse donor populations, together with comprehensive molecular validation and clinical outcome assessments, are warranted to confirm and extend these findings.

### Conclusion

In this study, the PCR-SSP technique was employed, which is particularly valuable to identify Di<sup>b</sup> antigen status in the absence of specific antisera; thereby, facilitating accurate identification of rare Di(a+b-) phenotypes in this cohort (approximately 1 in 3,500). This molecular approach may facilitate the identification of compatible donors and could support transfusion safety, although clinical outcomes were not evaluated in the present study.

### Ethical approval

This cross-sectional study was approved by the Human Research Ethics Committee of Thammasat University Hospital (HREC-TUH, COE No. 015/2569).

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### Conflict of interest

The authors declare they have no conflict of interest.

### CRediT authorship contribution statement

**Sarisa Chidtrakoo:** data and sample collection, method; **Wiradee Sasikarn:** method; **Tanaporn Choychimplee:** method, statistical analysis; **Kamphon Intharanut:** data interpretation, writing and review; **Oytip Nathalang:** conceptualization, method investigation, data interpretation, writing: review and editing.

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