

Genotyping Approach to Predict Co^a and Co^b Antigens in Thai Blood Donor Populations

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Purpose: Co^a and Co^b antigens of the Colton (CO) blood group system are implicated in acute and delayed hemolytic transfusion reactions (HTRs). Owing to the inadequate supply of specific antiserum, data on CO phenotypes remain limited. This study aimed to develop genotyping methods to predict Co^a and Co^b antigens and to estimate transfusion-induced alloimmunization risks in three Thai blood donor populations.

Materials and Methods: The study included 2451 blood samples from unrelated healthy Thai blood donors obtained from central, northern, and southern Thailand. DNA sequencing was used to determine the *CO*A* and *CO*B* alleles. In-house PCR with sequence-specific primers (PCR-SSP) and high-resolution melting curve (HRM) assays were performed and genotyping results were compared using DNA sequencing. *CO*A* and *CO*B* allele frequencies among Thais were determined using PCR-SSP and their frequencies were compared with other populations. The risks of Co^a and Co^b transfusion-induced alloimmunization among Thai donor populations were calculated.

Results: The validated genotyping results by PCR-SSP and HRM assays agreed with DNA sequencing. The *CO*A/CO*A* was the most common (100.0, 100.0, and 99.3%), followed by *CO*A/CO*B* (0.0, 0.0, and 0.7%) among central, northern and southern Thais. Homozygous *CO*B/CO*B* was not found. The *CO*A* and *CO*B* allele frequencies among central Thais significantly differed compared among southern Thais ($p < 0.01$) but not among northern Thais. Those allele frequencies among Thais were similar to those of Taiwanese, Chinese and Malay-Malaysian populations but not to South Asian, Southeast Asian, Korean, Japanese, Filipino, French Basque, and Maltese populations ($p < 0.01$). A higher risk of anti-Co^b production rather than anti-Co^a production was particularly noted in the southern Thai population.

Conclusion: This study constitutes the first to determine *CO*A* and *CO*B* genotypes using PCR-SSP and HRM assays among Thais and this finding would be beneficial in predicting alloimmunization risk and providing safe transfusions among Thais.

Keywords: the Colton blood group system, genotypes, PCR-SSP, HRM, Thais

Introduction

The Colton (CO) blood group system (ISBT No. 015) currently consists of four antigens; a high prevalence antigen, Co^a with its antithetical antigen, Co^b, and the other high prevalence antigens, Co3 and Co4.¹ The CO antigens are carried on red blood cell (RBC) water transporter, aquaporin-1 (AQP1), encoded by the *AQP1* gene located on chromosome 7p14.^{2,3} Regarding the frequency of Co^a and Co^b antigens in populations, Co(a+b-) is the most common phenotype, 91.4–100.0%, followed by Co(a+b+), 0.0–8.4%, and Co(a-b+), 0.0–0.2%. The Co(a-b-) is a rare phenotype.^{4–7} Anti-Co^a is implicated in both acute and delayed hemolytic transfusion reactions (HTRs), and mild to severe hemolytic diseases of the fetus and newborn (HDFN). In contrast, anti-Co^b, a rare antibody, has been involved in acute and delayed HTRs, while no case of HDFN has been reported.^{2–4}

Both anti-Co^a and anti-Co^b are commonly IgG and reactive by the indirect antiglobulin test (IAT), especially when using protease-treated RBCs.^{3,8} However, IgM anti-Co^a has been reported.^{3,4} To date, anti-Co^a and anti-Co^b are not commercialized and are only available in specialized reference laboratories. Moreover, some limitations to perform precise Co^a and Co^b antigen typing using serological techniques are due to the positive direct antiglobulin test (DAT) and positive autocontrol in frequent transfused patients.² On the other hand, patients with multiple alloantibodies or unidentified antibodies make it challenging to determine antibody specificities; as a result, additional panel cells with uncommon or low prevalence antigens such as Kp^a, Vel and Co^b are needed.⁹

Given that antiserum for CO phenotyping is unavailable, CO genotyping can help to select the most appropriate blood for patients with anti-Co^a or anti-Co^b. The Co^a/Co^b polymorphism arises from a single nucleotide variation (SNV) associated with the c.134C>T (rs28362692) missense variant encoding p.Ala45Val (alanine for Co^a and valine for Co^b) in exon 1 of the *AQP1* gene.²⁻⁴ Polymerase chain reaction with sequence-specific primers (PCR-SSP) was implemented to distinguish between *CO*A* and *CO*B* alleles to predict Co^a and Co^b antigens among unrelated French Basque and Maltese blood donors.^{10,11} Additionally, multiplex PCR has also been used to identify those alleles in Austrian blood donor populations.¹² Subsequently, commercially available high-throughput techniques for red cell genotyping have been performed only in reference laboratories due to the high cost of reagents and the required complex equipment.^{5,6,13}

In Thailand, low-throughput methods such as PCR-SSP and multiplex PCR were performed to predict extended blood group phenotypes in donor and patient populations, and the results were consistent with the serological testing.¹⁴ Recently, high-resolution melting curve (HRM) analysis has been implemented to reduce errors in interpretation and manual inspection for red cell genotyping using PCR-SSP.¹⁵ Therefore, this study aimed to develop genotyping methods to predict Co^a and Co^b antigens and to estimate transfusion-induced alloimmunization risks in three Thai blood donor populations.

Materials and Methods

Sample Size

This experimental cross-sectional study was approved by the Human Research Ethics Committee of Thammasat University (HREC-TUSc) Pathumthani, Thailand in accordance with the Declaration of Helsinki (COE No. 012/2565). Written informed consent was obtained from all individual participants. The study included 2451 samples of EDTA-anticoagulated blood from unrelated healthy Thai blood donors. In all, 1724 samples were from the National Blood Centre, Thai Red Cross Society, Bangkok, Thailand. In addition, 300 samples were from the Blood Bank Section, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand and 427 samples were from the Regional Blood Centre, 12th Songkhla, Thai Red Cross Society, Songkhla, Thailand.

Genomic DNA, extracted from all subjects using a Genomic DNA extraction kit (REAL Genomics, RBCBioscience, Taipei, Taiwan), was then stored at -20°C until used for genotyping.

Sequence-Based Genotyping

The genomic DNA of 400 blood donors was sequenced to identify *CO*A* and *CO*B* alleles. Primers were designed using Primer3 and BLAST. A fragment of 150 bp containing SNV (c.134 C>T; p. Ala45Val) in exon 1 of the *AQP1* gene was obtained from PCR amplification of genomic DNA using SEQ-CO-150-F forward and SEQ-CO-150-R reverse primers (Table 1). For each PCR reaction, 2 µL of genomic DNA (50 ng/µL) was a template for PCR amplification as a starting amount in a total volume of 50 µL using 0.6 µM of both forward and reverse primers for each reaction. PCR was performed using 25 µL of 2X PCR reaction mixture (Phusion High-Fidelity PCR Master Mix, New England BioLabs, MA, USA) and 17 µL of sterile distilled water in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

PCR was performed under the following conditions. Initial denaturation was conducted at 95°C for 30 sec. The PCR program began with 10 cycles of 10 sec at 95°C and 60 sec at 69°C, then 20 cycles of 30 sec at 95°C, 60 sec at 62°C, and 30 sec at 72°C. The last step was a final extension for 5 min at 72°C. The PCR products were subjected to electrophoresis and were visualized under a blue-light transilluminator. Then the PCR products were purified using a gel extraction kit

Table 1 Sequences of the Primers for *CO**A and *CO**B Genotyping

Specificity	Primer	Primer Sequences (5'→3')	Product Size (bp)	Uses
CO	SEQ-CO-150-F	GCCACGACCCTCTTGTCTT	150	PCR amplification, Sequencing, HRM
	SEQ-CO-150-R	GTGGCCACACTCTGCG		
CO *A	CO-A-F	GGGGAACAACCAGACGGC	225	PCR-SSP
	CO-225-R	CCTGAGAGGATGGCGGTG		
CO *B	CO-B-F	GGGGAACAACCAGACGGT	225	PCR-SSP
	CO-225-R	CCTGAGAGGATGGCGGTG		
HGH	HGH-F	TGCCTTCCCAACCATTCCCTTA	434	PCR-SSP
	HGH-R	CCACTCACGGATTCTGTTGTGTTTC		

Abbreviations: Bp, base pair; CO, Colton; HGH, human growth hormone; HRM, high-resolution melting curve; PCR, polymerase chain reaction; PCR-SSP, PCR-sequence-specific primer.

(GeneJET Gel Extraction Kit, Thermo Scientific, MA, USA), and eluted fragments were sequenced (U2Bio Sequencing Service, Bangkok, Thailand) using the abovementioned PCR primers.

PCR-SSP

To identify *CO**A and *CO**B alleles the in-house PCR-SSP technique was used. Primers and amplification conditions were designed (Table 1). Briefly, 1 µL of genomic DNA (50 ng/µL) was a template for PCR amplification as a starting amount in a total volume of 10 µL using 0.5 µM of CO-A-F and CO-225-R primers to detect *CO**A alleles, and 0.5 µM of CO-B-F and CO-225-R primers to detect *CO**B alleles. Co-amplification of the *human growth hormone (HGH)* gene using 0.6 µM of HGH-F and HGH-R primers was run as the internal control. The PCR was performed with 5 µL of 2 x PCR reaction mixture (OnePCR Plus, GeneDirex, Taiwan) in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using the identical abovementioned PCR conditions, and the sample was kept at 4°C. PCR products were subjected to electrophoresis at 100 volts on 1.5% agarose gel using 1X TBE buffer and were visualized under a blue-light transilluminator. The PCR product size of both *CO**A and *CO**B alleles was 225 bp, whereas that of the internal control, the *HGH* gene, was 434 bp. Known DNA controls of *CO**A/*CO**A, and *CO**A/*CO**B genotypes were used as positive and negative controls in parallel with unknown samples. Altogether, 2451 DNA samples (including 400 samples tested by DNA sequencing), were used to detect *CO**A and *CO**B alleles using PCR-SSP. Additionally, the DNA samples from 100 randomly genotyped blood donors were retested by PCR-SSP with identical conditions as the first round testing and sequenced to validate our in-house PCR-SSP technique for CO genotyping.

HRM Assay

The HRM assay was developed for CO genotyping in 400 DNA samples confirmed by DNA sequencing. Each pair of forward and reverse primers SEQ-CO-150-F and SEQ-CO-150-R was designed to amplify the CO genes (Table 1), and 150 bp PCR products were expected. A DNA typing kit (Type-it HRM PCR Kit, QIAGEN GmbH, Valencia, CA, USA) was used to perform the PCR-HRM assay.

In a PCR reaction mix, 10 µL of 2X HRM PCR Master Mix reagent, 1 µL of genomic DNA (10 to 50 ng/µL concentration), and 0.35 µM of both forward and reverse primers were combined with water to make a final volume of 20 µL. PCR reaction tubes were placed on a real-time PCR cycler (Rotor-Gene Q 5plex HRM, QIAGEN GmbH, Valencia, CA, USA). The cycling parameters for the PCR program consisted of initial denature at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, 62°C for 40 sec and 72°C for 30 sec. At the end of the PCR step, the temperature was gradually increased by 0.1°C every 2 sec from 65°C to 95°C. Total fluorescence and the rate of change in fluorescence were monitored, and data were acquired on the green and HRM channels. Rotor-Gene Q Series Software was used to analyze

the HRM data. Unknown melting profiles of DNA samples were compared with a set of determined controls on an allele-target basis. A confidence threshold of 90% was set for HRM genotyping calls.

To increase the validity and reliability of the evaluation, technicians were blinded to the PCR-SSP results. DNA sequencing was performed in case any discrepant genotyping results were obtained using the HRM and PCR-SSP assays.

Statistical Analysis

The gene and allele frequencies among the Thai blood donors were calculated using the gene-counting method. The chi-squared (χ^2) test was used to evaluate whether the observed genotype frequencies agreed with the expected ones under the Hardy-Weinberg equilibrium. The two-tailed Fisher's exact test of homogeneity was used to determine the differences of their allele frequencies among central, northern and southern Thais and to compare the differences with those reported in other populations.^{6,7,10,11,16,17} A *p*-value of less than 0.05 was considered statistically significant.

Results

Sequencing studies on 400 DNA Thai blood donor samples that exhibited the *CO**A** allele revealed the presence of c.134C, while the c.134T represented the *CO**B** allele. The two patterns of sequencing chromatograms are shown in Figure 1. The distribution of genotyping results among 400 DNA samples revealed that 398 were homozygous *CO**A*/CO**A**, and 2 were heterozygous *CO**A*/CO**B**. Homozygous *CO**B*/CO**B** was not found in this group.

The results of the PCR-SSP were used to distinguish between the *CO**A** and *CO**B** alleles. The first and second mixes could identify the *CO**A** and *CO**B** alleles with an amplified product size of 225 bp, as shown in Figure 2. The validated genotyping results by PCR-SSP among DNA controls (*CO**A*/CO**A** and *CO**A*/CO**B**) were consistent with each other, and the results in both sets showed 100% concordance with 400 known DNA sequencing results. In addition, repeated

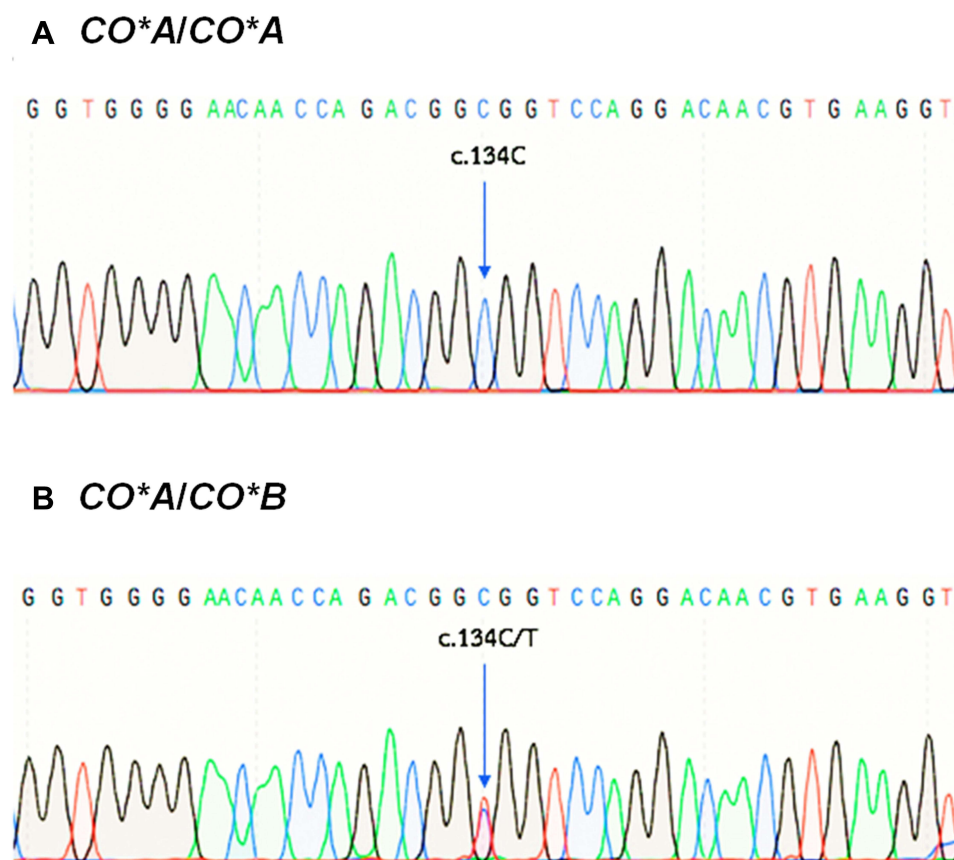


Figure 1 Electropherograms of the AQP1 Gene at the CO*A and CO*B Polymorphism Region, SNV c.134C>T (rs28362692). (A) Homozygous state of the c.134C identified in the CO*A/CO*A genotype and (B) Heterozygous state of the c.134C/T identified in the CO*A/CO*B genotype.

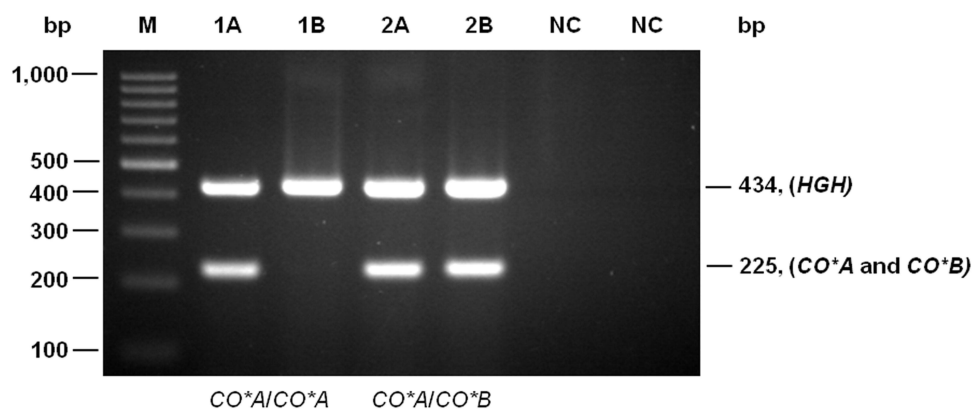


Figure 2 Representative Gel Shows Genotyping Results of the *CO* A* and *CO* B* Alleles Using the PCR-SSP Technique. The 434 bp amplification product of the HGH control primers was present in all lanes, showing that amplification had occurred optimally. The genotype was deduced from the presence or absence of 225 bp amplification product specific for *CO* A* and *CO* B* alleles. Lanes A and B referred to the specific CO allele test (*CO* A* and *CO* B*, respectively). From left to right: Lanes 1A-1B (SM101) = *CO* A/CO* A*; 2A-2B (SM106) = *CO* A/CO* B* and NC = negative control, respectively. M: 100 bp ladder marker (Fermentas, Carlsbad, CA, USA).

PCR-SSP was performed using 100 random DNA samples and confirmed by DNA sequencing. Those results of repeated testing were identical to the first round of testing and agreed with DNA sequencing.

Altogether, 1724, 300, and 427 samples obtained from central, northern and southern Thai blood donors, respectively, were genotyped for *CO* A* and *CO* B* separately, using the developed PCR-SSP (Table 2). *CO* A/CO* A* was found to be the most common genotype in the three groups of Thai blood donors (100.0, 100.0, and 99.3% in central, northern and southern Thais, respectively), while *CO* A/CO* B* was found only in southern blood donors (3/427, 0.7%); *CO* B/CO* B* was not found in the three groups. The allele frequencies of *CO* A* in central, northern and southern Thais were 1.000 (3448/3448), 1.000 (600/600), and 0.996 (851/854), respectively. In addition, the frequencies of *CO* B* were 0.000 (0/3448), 0.000 (0/600), and 0.004 (3/854), respectively. The genotypes of the 1724 central, 300 northern and 427 southern Thais were consistent with each other according to the Hardy-Weinberg equilibrium. The detected *CO* A* and *CO* B* allele frequencies among Thai blood donors significantly differed among central Thais compared with southern Thais ($p < 0.01$) but not among northern Thais (Table 2). Moreover, PCR-SSP was performed using 100 randomly selected samples consisting of 98 homozygous *CO* A/CO* A*, and two heterozygous *CO* A/CO* B*, confirmed by DNA sequencing. The results of the repeated testing were consistent with the DNA sequencing results.

DNA controls of *CO* A/CO* A* and *CO* A/CO* B* were amplified and analyzed using the HRM assay, while *CO* A* and *CO* B* alleles were genotyped using HRM curves. Representative normalized HRM curves were of two types: the red curve representing the homozygous *CO* A/CO* A* genotypes and the purple curve representing the heterozygous *CO* A/CO* B* genotypes. HRM curves showed a clear separation in those two genotypes at T_m ranging from 86.5 to 87.5°C for the CO genotyping (Figure 3). The developed HRM assay was implemented in 400 donor samples to predict phenotypes

Table 2 *CO* A* and *CO* B* Genotype and Allele Frequencies Among Thai Blood Donors

Frequency	Central Thais (N = 1724)	Northern Thais (N = 300)	Southern Thais (N = 427)	Total (N = 2451)
Allele				
<i>CO* A</i>	3448 (1.0000)	600 (1.0000)	851 (0.9965)	4899 (0.9994)
<i>CO* B</i>	0 (0.0000)	0 (0.0000)	3 (0.0035)	3 (0.0006)
Genotype				
<i>CO* A/CO* A</i>	1724 (100.0)	300 (100.0)	424 (99.3)	2448 (99.9)
<i>CO* A/CO* B</i>	0 (0.0)	0 (0.0)	3 (0.7)	3 (0.1)
<i>CO* B/CO* B</i>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Note: Allele frequencies in bold differed from those among central Thai blood donors ($p < 0.05$).

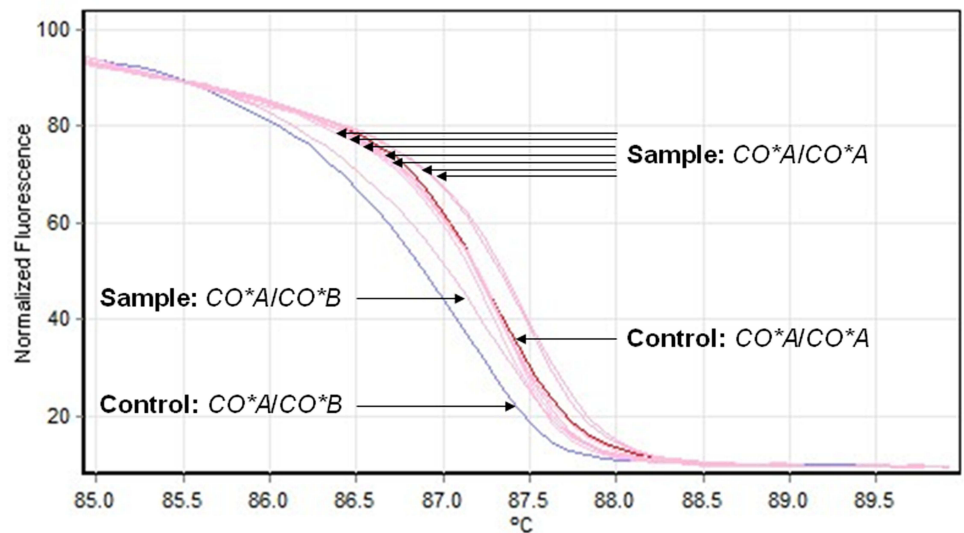


Figure 3 HRM analysis of *CO* genotypes.

of Colton blood group systems. The results were concordant with those 400 samples obtained for genotypes by DNA sequencing and PCR-SSP.

The *CO***A* and *CO***B* allele frequencies in Thai and other populations are shown in Table 3. The *CO***A* and *CO***B* frequencies among Thais were similar to previously reported in Thai,¹⁶ Taiwanese,¹⁷ Chinese⁶ and Malay-Malaysian⁷ populations ($p > 0.05$). Significant differences were observed when *CO***A* and *CO***B* allele frequencies among Thais

Table 3 Comparison of the *CO***A* and *CO***B* Allele Frequencies Among Different Populations

Population	Number of Alleles	Allele Frequency		<i>p</i> -value	Methods
		<i>CO</i> * <i>A</i>	<i>CO</i> * <i>B</i>		
Thai (current study)	4902	0.9994	0.0006	NA	PCR-SSP
Thai ¹⁶	790	0.9987	0.0013	0.45	MALDI-TOF MS
Thai ¹⁷	420	1.0000	0.0000	1.00	PCR-RFLP
Taiwanese ¹⁷	800	1.0000	0.0000	1.00	PCR-RFLP
South Asian ⁶	1844	0.9930	0.0070	< 0.0001	DNA array
Southeast Asian ⁶	1884	0.9965	0.0035	0.0066	DNA array
Chinese ⁶	3430	0.9985	0.0015	0.2867	DNA array
Korean ⁶	2066	0.9960	0.0040	0.0039	DNA array
Japanese ⁶	2044	0.9920	0.0080	< 0.0001	DNA array
Malay-Malaysian ⁷	194	0.9948	0.0052	0.1438	PCR-SSP
Filipino ⁶	2666	0.9950	0.0050	0.0002	DNA array
French Basque ¹⁰	222	0.9640	0.0360	< 0.0001	PCR-ASP
Maltese ¹¹	136	0.9412	0.0588	< 0.0001	PCR-ASPE

Note: Allele frequencies in bold differed from those among Thai blood donors ($p < 0.05$).
Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NA, Not applicable; PCR-ASP, polymerase chain reaction-allele-specific primer; PCR-ASPE, PCR-allele-specific primer extension; PCR-RFLP, PCR- restriction fragment length polymorphism; PCR-SSP, PCR-sequence-specific primer.

compared with South Asian,⁶ Southeast Asian,⁶ Korean,⁶ Japanese,⁶ Filipino,⁶ French Basque,¹⁰ and Maltese¹¹ populations ($p < 0.01$).

The risks of Co^a and Co^b alloimmunization in the three Thai populations were calculated, according to the observed phenotypes. Because the *CO*B/CO*B* genotype was not found in all Thai populations, only the alloimmunization risk of anti-Co^b production is supposed to be calculated. The *CO*A/CO*B* genotype was only observed in southern Thais; hence, the possibility of anti-Co^b production was 0.0069, estimated by *CO*A/CO*B* donor to *CO*A/CO*A* patient (0.9930×0.0070). Regarding regional groups, the risk of Co^b alloimmunization in southern Thailand was higher than in central and northern Thailand.

Discussion

For the Colton blood group system, anti-Co^a and anti-Co^b are required to determine the three CO phenotypes. To overcome the limitation of specific antisera, red cell genotyping is helpful in predicting phenotypes among blood donors and patients. Moreover, it remains impossible to identify various antibody specificities when employing reagent RBCs with only limited serologically antigen-typed information. A related study revealed the impact of using genotyped RBCs to identify antibody specificities. Altogether, 21 different antibodies from 10 different blood group systems including Knops, Yt, Vel, Dombrock, Lutheran, Rh, Diego, Colton, Scianna and Landsteiner-Wiener could be identified among 126 patients (1.5%) due to the genotyped information of the reagent RBCs.⁹

Red cell genotyping by commercially available Beadchip array has been used for assigning 34 antigens in 11 blood group systems in different populations.⁶ Nevertheless, some blood group antigens might only be significant in somewhat different populations and the reagents required are becoming costly. In this study, we developed the genotyping methods to determine *CO*A* and *CO*B* alleles, and PCR-SSP and HRM genotyping results agreed with DNA sequencing results; hence, the genotyping results were accurate and reproducible.

In addition, *CO* allele frequencies of Thai populations were compared with those reported of other groups.^{6,7,10,11,16,17} The significant difference in *CO*A* and *CO*B* frequencies among Thais compared with French Basque and Maltese populations might induce a higher probability of finding anti-Co^a in Co(a-b+) patients than in Thai patients. However, the rare donors, Co(a-b+) phenotype, still exist among those populations.^{10,11} A similar prevalence of predicted Co(a+) phenotype among Thais and Chinese was found in this study, corresponding to the Do(a+) phenotype in the Dombrock blood group system.¹⁸ Genotyping methods such as PCR-SSP and HRM are applicable to predict the phenotypes of extended blood group systems.^{14,15,19}

In Thailand, the Co^a/Co^b phenotyping is not routinely performed, and reagent red cells for antibody identification are also unspecified Co^a/Co^b antigens. In this study, the predicted Co^a and Co^b antigens were obtained by genotyping results, the highest prevalence was Co(a+b-) phenotype in all three Thai populations and Co(a+b+) donors were only observed in southern Thais. The possibility of anti-Co^b production caused by blood transfusion is high, especially among southern Thais compared with central and northern Thais. However, a related study of frequencies and antibody specificities among alloimmunized southern Thai patients revealed that more than 5% were undetermined antibody specificities.²⁰ Anti-Co^b may therefore hide in those patients owing to unspecified Co^a/Co^b antigens in panel cells. The genotyping in reagent RBCs, in particular Co(b+), is crucial to identify antibody specificity to prevent adverse transfusion reactions and to screen appropriate blood donors among patients with either anti-Co^a or anti-Co^b. Although the Co(a-b+) phenotype was not observed in this study, a patient with this phenotype produces an anti-Co^a, autologous blood donation, and recruiting related donors will be necessary.

Conclusion

This study constitutes the first to determine *CO*A* and *CO*B* genotypes in Thai populations using in-house PCR-SSP and HRM assays. This finding would be beneficial in predicting the risk of alloimmunization, especially anti-Co^b production, and providing safe transfusions among Thais.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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