Gene frequencies of the HPA-1 to 6 and Gov human platelet antigens in Thai blood donors

P. KUPATAWINTU, O. NATHALANG, R. O-CHAROEN, AND P. PATMASIRIWAT

Human platelet alloantigens (HPA) are important in neonatal alloimmune thrombocytopenia (NAIT), posttransfusion purpura (PTP), platelet transfusion refractoriness, passive alloimmune thrombocytopenia, and transplantation-associated alloimmune thrombocytopenia. Thus, *HPA* genotyping is essential in diagnosis and treatment. We analyzed *HPA-1* to *6* and *Gov* alleles, using PCR with sequence specific primers (PCR-SSP) in 500 Thai blood donors who had been HLA class I antigen typed. *HPA-4a* was present in all samples. *HPA-1b, -2b, -5b,* and *-6b* were rare, and *HPA-4b* was not found. *HPA-3a* and *-3b* showed frequencies of 56.0 percent and 44.0 percent, respectively. *Gov*^{a} and *Gov*^{b} showed frequencies of 49.1 percent and 50.9 percent, respectively. The prevalence rates of *HPA-1* to *6* gene frequencies (GFs) were consistent with those of other Asian populations rather than those of Caucasians. We also report on the GFs of *Gov*^{a} and *Gov*^{b}, which also are comparable to those of Asian populations. Our results could establish a useful HPA- and HLA-matched plateletpheresis donor file and provide an improvement of platelet alloantibody detection in alloimmune thrombocytopenic patients, and, therefore, a more effective platelet transfusion program. ***Immunohematology* 2005;21:5–9.**

Key Words: human platelet antigen (HPA), Gov, PCR-SSP, Thais, gene frequencies

Human platelet alloantigens (HPA) become clinically relevant if the antibody causes enhanced platelet destruction, resulting in thrombocytopenia and hemorrhagic diathesis. Five clinical entities due to platelet-specific alloantibodies can be distinguished: neonatal alloimmune thrombocytopenia (NAIT), post-transfusion purpura (PTP), platelet transfusion refractoriness, passive alloimmune thrombocytopenia, and transplantation-associated alloimmune thrombocytopenia.[1] Platelet transfusion refractoriness, which is defined as a low CCI after platelet transfusion, is a common problem in multitransfused patients. Immunologic refractoriness to platelet transfusion is caused by alloantibodies reacting with transfused antigens such as ABO, HLA class I, and HPA antigens. Although HLA alloimmunization is the most common, HPA antibodies are also clinically significant.[1–4]

In Thailand, platelet antigen typing of blood donors is not routinely performed, except for population genetic studies.[5–7] Usually, ABO matched and negative platelet crossmatched units are transfused to multitransfused patients with platelet refractoriness. The National Blood Centre, Thai Red Cross Society, has performed platelet antibody screening and identification in 163 thrombocytopenic patients, using the solid phase red cell adherence assay, since 1993. HLA and HPA antibodies were detected in 49.63 percent and 6.13 percent of patients, respectively. In addition, 40.0 percent of HPA antibodies could not be identified due to the limited availability of HPA-genotyped panels.[8] Having a panel of HLA- and HPA-typed donors would be helpful.[4] Different PCR techniques have been introduced, such as PCR with RFLP and PCR with sequence specific primers (PCR-SSP). The PCR-SSP technique has been shown to be a simpler and more reliable method for several HPA genotypes.[9–14]

This study analyzed *HPA-1* to *6* and *Gov* alleles, using PCR-SSP in Thai blood donors who had been HLA class I antigen typed to establish HPA-genotyped panels for identification of HPA antibodies in routine testing.

Materials and Methods

Subjects

Five hundred healthy Thai blood donors at the National Blood Centre, Thai Red Cross Society, who had been HLA class I antigen typed, were included in this study. The study comprised 369 males and 131 females, with ages ranging from 19 to 58 years. The mean age was 36 years. EDTA blood (3 mL) was collected from each donor and informed consent was obtained from all subjects.

DNA standards

Known *HPA-1* to *6* and *Gov* DNA samples, provided by the Australian Platelet Antibody Workshop in association with the Australian and New Zealand Society of Blood Transfusion and Central Blood Centre, Japanese Red Cross Society, were used as a standard in the PCR-SSP method.

HPA genotyping

Genomic DNA was extracted from whole blood by the salting-out technique (Pel-Freez, Brown Deer, WI). The PCR-SSP for *HPA-1* to *6* and *Gov* systems was performed as previously described,[9,15,16] with some modification. To increase specificity for detection of HPA-3b, two bases were extended at the 5´ end of the primer. The primers used in this study are listed in Table 1. Briefly, the PCR reactions were carried out in 10 µL aliquots containing 50–100 ng of genomic DNA and PCR buffer (67 mM Tris HCL pH 8.8, 16 mM ammonium sulfate, 0.01% Tween 20, 0.5 µM each of dNTP and 1.5 mM $MgCl_2$). Each PCR reaction contained 0.1 to 0.4 µM of the control primers, 0.1 to 0.5 µM of the allele-specific primers, and 0.75 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). PCR amplifications were carried out in a GeneAmp PCR System 2700 (Applied Biosystems) or in a PTC-200 (MJ Research, Waltham, MA). The PCR program consisted of one cycle: 96°C for 1 minute; five cycles: 96°C for 25 seconds, 68°C for 45 seconds, 72°C for 30 seconds; 28 cycles: 96°C for 25 seconds, 61°C for 45 seconds, 72°C for 30 seconds; and one cycle: 72°C for 3 minutes. The amplified products were electrophoresed through 2.0% agarose gel containing 0.5 µg/mL ethidium bromide. The gel was run at 100 V for 30 minutes in 0.5X TBE and visualized under UV transilluminator. The reaction was photographed and the HPA alleles were assessed.

Reproducibility of SSP typing

Ten samples were randomly repeated for *HPA-1* to *6* and *Gov* SSP-typing, to test for reproducibility.

Statistical analysis

Genotype and gene frequencies (GF) were determined by direct counting. The validity of the Hardy-Weinberg equilibrium was tested by calculating expected numbers of subjects for each genotype using 2 × af[a] × af[b] × N for heterozygotes and af[a or b]2 × N for homozygotes, where af[a] and af[b] are the allele frequencies of the *HPA-1* to *6* and *Gov a* and *b* alleles, respectively, and N is the number of subjects typed. The differences in HPA genotype distribution between the two groups were tested for significance by chi-square and by Fisher's exact test.

Table 1. Sequence of the primers for HPA-1 to 6, Gov, and HGH internal control

Primer	Sequence	Product size (bp)	Final Conc.
HPA-1a	5´ TCA CAG CGA GGT GAG GCC A 3´		
HPA-1b	5´ TCA CAG CGA GGT GAG GCC G 3´	90	0.1 µM
HPA-1 common	5´ GGA GGT AGA GAG TCG CCA TAG 3´		
HPA-2a	5´ GCC CCC AGG GCT CCT GAC 3´		
HPA-2b	5´ GCC CCC AGG GCT CCT GAT 3´	258	0.1 µM
HPA-2 common	5´ TCA GCA TTG TCC TGC AGC CA 3´		
HPA-3a	5´ TGG ACT GGG GGC TGC CCA T 3´	267	
HPA-3b	5´ GGT GGA CTG GGG GCT GCC CAG 3´	269	0.2 µM
HPA-3 common	5´ TCC ATG TTC ACT TGA AGT GCT 3´		
HPA-4a	5´ GCT GGC CAC CCA GAT GCG 3´		
HPA-4b	5´ GCT GGC CAC CCA GAT GCA 3´	120	0.1 µM
HPA-4 common	5´ CAG GGG TTT TCG AGG GCC T 3´		
HPA-5a	5´ AGT CTA CCT GTT TAC TAT CAA AG 3´		
HPA-5b	5´ AGT CTA CCT GTT TAC TAT CAA AA 3´	246	0.5 µM
HPA-5 common	5´ CTC TCA TGG AAA ATG GCA GTA 3´		
HPA-6a	5´ GAC GAG TGC AGC CCC CG 3´	238	
HPA-6b	5´ GGA CGA GTG CAG CCC CCA 3´	239	0.2 µM
HPA-6 common	5´ CTA TGT TTC CCA GTG GTT GCA 3´		
Gov[a]	5´ TTC AAA TTC TTG GTA AAT CCT GT 3´		
Gov[b]	5´ TTC AAA TTC TTG GTA AAT CCT GG 3´	225	0.4 µM
Gov common	5´ ATG ACC TTA TGA TGA CCT ATT C 3´		
HGH control	5´ GCC TTC CCA ACC ATT CCC TTA 3´		HPA-1–4 & 6 0.4 µM
HGH control	5´ TCA CGG ATT TCT GTT GTG TTT C 3´	429	HPA-5 0.1 µM Gov 0.2 µM

Results

In this study, the simultaneous determination of *HPA-1* to *6* and *Gov* genotyping by PCR-SSP resulted in PCR products of the following sizes: 90 bp for *HPA-1,* 258 bp for *HPA-2,* 267 and 269 bp for *HPA-3a* and *-3b,* 120 bp for *HPA-4,* 246 bp for *HPA-5,* 238 and 239 bp for *HPA-6a* and *-6b,* and 225 bp for *Gov,* respectively. In all reactions, the human growth hormone (HGH) internal control gave an expected band of 429 bp. Additional smaller, nonspecific products were seen in *HPA-3b* and *-6b,* while a larger nonspecific product was also seen in *HPA-4b,* as previously reported,[4] but these did not interfere with the interpretation of the HPA genotypes (Fig. 1).

The genotype and gene frequencies of the seven platelet antigen systems obtained from 500 Thai blood

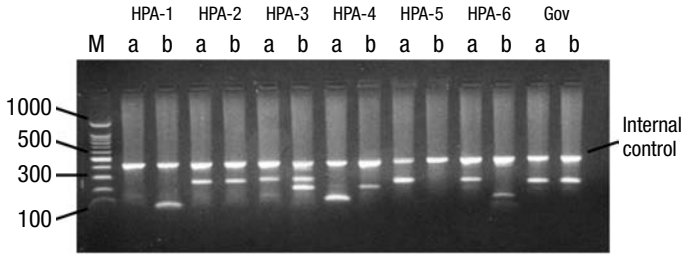

Fig. 1. A representative gel showing simultaneous *HPA-1* to *6* and *Gov* genotyping by PCR-SSP. The 429-bp amplification product of the HGH control primers is present in all lanes, which shows that amplification has occurred optimally. The genotype was deduced from the presence or absence of amplification products specific for alleles (from left to right: *HPA-1b1b, HPA-2a2b, HPA-3a3b, HPA-4a4a, HPA-5a5a, HPA-6a6a, Gov-a/b*). M: 100-bp ladder marker (Promega, Madison, WI).

donors are shown in Table 2. *HPA-4a* was present in all samples. *HPA-1b, -2b, -5b,* and *-6b* were rare and *HPA-4b* was not found. *HPA-3a* and *-3b* showed frequencies of 56.0 percent and 44.0 percent, respectively. Gov^a and Gov^b showed frequencies of 49.1 percent and 50.9 percent, respectively.

To test the reproducibility of the assay, ten DNA samples were randomly selected and tested for *HPA-1* to *6* and *Gov* typing. The results of the repeated assay were similar to those of the first round of testing. Moreover, this PCR-SSP HPA typing method was validated using 20 HPA reference materials typed by alternative techniques and in other laboratories. The results showed a 100 percent concordance between the two methods.

A comparison of *HPA-1* to *6* and *Gov* gene frequencies among Asian and Caucasian studies is presented in Tables 3 and 4.[6,12,17–23] The *HPA-1* to *6* frequencies in Thai blood donors are more similar to those in Northeastern Thais and other Asians than to those of Caucasians. The Gov^a and Gov^b frequencies in our study showed results similar to those in the previous study[23] but were significantly different from those in the study in UK populations ($p < 0.01$).[24]

Table 2. The genotype and gene frequencies of *HPA-1 to 6* and *Gov* in 500 Thai blood donors

Genotype	No.	Genotype frequencies (%)	Gene	Gene frequencies (%)
HPA-1a1a	485	97.0	*HPA-1a*	98.5
HPA-1a1b	15	3.0	*HPA-1b*	1.5
HPA-1b1b	0	0.0		
HPA-2a2a	452	90.4	*HPA-2a*	95.2
HPA-2a2b	48	9.6	*HPA-2b*	4.8
HPA-2b2b	0	0.0		
HPA-3a3a	152	30.4	*HPA-3a*	56.0
HPA-3a3b	256	51.2	*HPA-3b*	44.0
HPA-3b3b	92	18.4		
HPA-4a4a	500	100.0	*HPA-4a*	100.0
HPA-4a4b	0	0.0	*HPA-4b*	0.0
HPA-4b4b	0	0.0		
HPA-5a5a	468	93.6	*HPA-5a*	96.8
HPA-5a5b	32	6.4	*HPA-5b*	3.2
HPA-5b5b	0	0.0		
HPA-6a6a	486	97.2	*HPA-6a*	98.6
HPA-6a6b	14	2.8	*HPA-6b*	1.4
HPA-6b6b	0	0.0		
Gov^a/Gov^a	125	25.0	Gov^a	49.1
Gov^a/Gov^b	241	48.2	Gov^b	50.9
Gov^b/Gov^b	134	26.8		

Discussion

The investigation of *HPA* gene and genotype frequencies is important in both population studies

Table 3. Gene frequencies of *HPA-1* to *6* in 500 Thai blood donors and different populations

HPA	Thai* (N=500)	NET† (N=300)	Taiwan[17] (N=300)	Hong Kong[18] (N=100)	Korea[19] (N=100)	Japan[20] (N=331)	Spain[21] (N=107)	Wales[12] (N=392)	Australia[22] (N=1,000)	Norway[23] (N=105)
-1a	98.5	97.2	99.7(a)	99.5	99.5	99.8(b)	74.8(c)	82.5(c)	85.8(c)	86.7(c)
-1b	1.5	2.8	0.3(a)	0.5	0.5	0.2(b)	25.2(c)	17.5(c)	14.2(c)	13.3(c)
-2a	95.2	93.8	96.0	97.5	87.0(c)	NA‡	81.8(c)	90.9(c)	92.7(b)	94.3
-2b	4.8	6.2	4.0	2.5	13.0(c)	NA	18.2(c)	9.1(c)	7.3(b)	5.7
-3a	56.0	53.3	57.5	52.5	67.0(b)	NA	68.2(b)	60.7(a)	61.9(b)	47.1(a)
-3b	44.0	46.7	42.5	47.5	33.0(b)	NA	31.8(b)	39.3(a)	38.1(b)	52.9(a)
-4a	100.0	100.0	99.8	100.0	100.0	98.9(b)	100.0	100.0	100.0	100.0
-4b	0.0	0.0	0.2	0.0	0.0	1.1(b)	0.0	0.0	0.0	0.0
-5a	96.8	96.3	98.5(a)	96.5	97.0	NA	86.1(c)	90.3(c)	90.5(c)	92.9(b)
-5b	3.2	3.7	1.5(a)	3.5	3.0	NA	13.9(c)	9.7(c)	9.5(c)	7.1(b)
-6a	98.6	98.5	96.3(b)	NA	NA	97.3	100.0	100.0(a)	NA	NA
-6a	1.4	1.5	3.7(b)	NA	NA	2.7	0.0	0.0(a)	NA	NA

*Thai blood donors †Northeastern Thais ‡No data available (a) $p < 0.05$ (b) $p < 0.01$ (c) $p < 0.001$

Table 4. Gene frequencies of *Gov* in 500 Thai blood donors and different populations

HPA	Thai* (N=500)	Norway[23] (N=105)	UK[24] (N=113)	Taiwan[25] (N=566)	Indonesia[25] (N=107)	Thai[25] (N=137)	Filipino[25] (N=100)
Gov[a]	49.1	49.5	40.0(b)	46.2	45.0	46.3	52.0
Gov[b]	50.9	50.5	60.0(b)	53.8	55.0	53.7	48.0

*Thai blood donors (b) $p < 0.01$

and clinical transfusion practice, where HPA-typed platelets may be required for alloimmunized patients. Providing more data on HPA distribution among various populations enables the prediction of the risk of platelet-specific alloimmunization in different ethnic groups and improves the ability to prepare HPA-compatible platelet products.[2,4]

Recent studies reported gene frequencies of the *HPA-1* to *13, Oe,* and *Gov* alleles in Taiwanese, Indonesian, Filipino, and Thai populations, using the PCR-RFLP technique.[11,25] Although the results were consistent with other previous findings in Asian populations, the PCR-RFLP technique has some disadvantages, such as being time-consuming and susceptible to being misinterpreted due to incomplete enzyme digestion of the amplicon.[16] In this study we have established *HPA-1* to *-6* and *Gov* genotyping by PCR-SSP, using a combination of established and modified sequence-specific primers. This method is rapid, cost-effective, and suitable in large-scale platelet antigen genotyping, either for NAIT or in genetic population studies.[4,6,9,10,12,14,17]

Furthermore, the distribution of HPA-1 to 6 phenotypes in Thai blood donors living in Bangkok is consistent with those found in Northeastern Thailand.[6,7] A previous study showed that the most common HPA antibodies in thrombocytopenic Thai patients were anti-HPA-5b, -HPA-2b, -HPA-3a, and unidentified antibodies. Anti-HPA-1a, which is the most common cause of NAIT and PTP in Caucasians, is not found in Thai populations due to the high frequency of HPA-1a (> 97%). Other studies showed that anti-HPA-1b, -HPA-2a, -HPA-2b, -HPA-3a, -HPA-3b, -HPA-4a, -HPA-4b, -HPA-5b,-HPA-5a, and -HPA-6a were also found in NAIT, PTP, and refractoriness to platelet transfusion therapy in Caucasians and in Japanese populations.[24,26–29]

The Gov antibodies have been reported for their clinical importance, especially in NAIT, PTP, and platelet refractoriness.[24,30] Although the incidence of Gov antibodies was lower than that of the HPA-1 system, the incidence was equal to that of HPA-5 system antibodies in Caucasians. The genotype frequencies of the Gov system in this study are comparable to those found in previous studies in Asian populations.[25] Thus, typing for the Gov antigen system should be useful in patient diagnosis. In summary, the implementation of the HPA genotyping system using the PCR-SSP technique in both donors and patients is beneficial in platelet transfusion therapy to provide HPA-matched platelet donors and to increase the capability for platelet alloantibody investigation.

Acknowledgments

We thank Dr. Paul Metcalfe, National Institute for Biological Standards and Control, UK, for his helpful advice and the Australian and New Zealand Society of Blood Transfusion and Central Blood Centre, Japanese Red Cross Society, for providing known *HPA-1* to *6* and *Gov* DNA samples.

References

1. Kroll H, Kiefel V, Santoso S. Clinical aspects and typing of platelet alloantigens. Vox Sang 1998; 74:345-54.
2. Kekomaki R. Use of HPA- and HLA-matched platelets in alloimmunized patients. Vox Sang 1998;74(Suppl 2):359-63.
3. Novotny AMJ. Prevention and management of platelet transfusion. Vox Sang 1999;76:1-3.
4. Verran J, Gray D, Bennett J, Lown JAG, Erber WN. HPA-1,3,5 genotyping to establish a typed platelet donor panel. Pathology 2000;32:89-93.
5. O-Charoen R, Kupatawintu P, Jitjak N. Human platelet-specific alloantigen: phenotype frequency in Thai blood donors: Preliminary report. Thai J Haematol Transfus Med 1993;3:27-35.
6. Romphruk AV, Akahat J, Srivanichrak P, Puapairoj C, Romphruk A, Leelayuwat C. Genotyping of human platelet antigens in ethnic Northeastern Thais by the polymerase chain reaction-sequence specific primer technique. J Med Assoc Thai 2000;83: 1333-7.
7. Urwijitaroon Y, Barusrux S, Romphruk A, Puapairoj C. Frequency of human platelet antigens among blood donors in Northeastern Thailand. Transfusion 1995;35:868-70.
8. Kupatawintu P, Jitjak N, Saelee S, O-Charoen R, Nathalang O. The incidence of platelet antibody in thrombocytopenic patients. Thai J Haematol Transfus Med 2000;10:123-7.

9. Cavanagh G, Dunn AN, Chapman CE. HPA genotyping by PCR sequence-specific priming (PCR-SSP): a streamlined method for rapid routine investigation. Transfus Med 1997;7:41-5.
10. Kluter H, Fehlau K, Panzer S, Kirchner H, Bein G. Rapid typing for human antigen systems-1, -2, -3 and –5 by PCR amplification with sequence-specific primers. Vox Sang 1996;71:121-5.
11. Liu TC, Shih MC, Lin CL, Lin SF, Chen CM, Chang JG. Gene frequencies of the HPA-1 to HPA-8w platelet antigen alleles in Taiwanese, Indonesian, and Thai. Ann Hematol 2002;81:244-8.
12. Sellers J, Thompson J, Guttridge MG, Darke C. Human platelet antigens: typing by PCR using sequence-specific primers and their distribution in blood donors resident in Wales. Eur J Immunogenet 1999;26:393-7.
13. Skogen B, Bellissimo DB, Hessner MJ, et al. Rapid determination of platelet alloantigen genotypes by polymerase chain reaction using allele-specific primers. Transfusion 1994;34:955-60.
14. Tanaka S, Taniue A, Nagao N, et al. Simultaneous DNA typing of human platelet antigens 2, 3 and 4 by an allele-specific PCR method. Vox Sang 1995; 68:225-30.
15. Hurd CM, Cavanagh G, Schuh A, Ouwehand WH, Metcalfe P. Genotyping for platelet-specific antigens: techniques for the detection of single nucleotide polymorphisms. Vox Sang 2002;83: 1-12.
16. Schuh AC, Watkins NA, Nguyen Q, et al. A tyrosine 703 serine polymorphism of CD109 defines the Gov platelet alloantigens. Blood 2002;99:1692-8.
17. Lyou JY, Chen YJ, Hu HY, Lin JS, Tzeng CH. PCR with sequence-specific primer-based simultaneous genotyping of human platelet antigen-1 to -13w. Transfusion 2002;42:1089-95.
18. Chang YW, Mytilineos J, Opelz G, Hawkins BR. Distribution of human platelet antigens in a Chinese population. Tissue Antigens 1998;51: 391-3.
19. Kim HO, Jin Y, Kickler TS, Blakemore K, Kwon OH, Bray PF. Gene frequencies of the five major human platelet antigens in African American, white, and Korean populations. Transfusion 1995;35:863-7.
20. Tanaka S, Ohnoki S, Shibata H, Okubo Y, Yamaguchi H, Shibata Y. Gene frequencies of human platelet antigens on glycoprotein IIIa in Japanese. Transfusion 1996;36:813-7.
21. Ferrer G, Muniz-Diaz E, Aluja MP, et al. Analysis of human platelet antigen system in a Moroccan Berber population. Transfus Med 2002;12:49-54.
22. Bennett JA, Palmer LJ, Musk AW, Erber WN. Gene frequencies of human platelet antigens 1-5 in indigenous Australians in Western Australia. Transfus Med 2002;12:199-203.
23. Randen I, Sorensen K, Killie MK, Kragh JK. Rapid and reliable genotyping of human platelet antigen (HPA)-1, -2, -3, -4 and –5 a/b and Gov a/b by melting curve analysis. Transfusion 2003;43:445-50.
24. Berry JE, Murphy CM, Smith GA, et al. Detection of Gov system antibodies by MAIPA reveals an immunogenicity similar to the HPA-5 alloantigens. Br J Haematol 2000;110:735-42.
25. Shin MC, Liu TC, Lin IL, Lin SF, Chen CM, Chang JG. Gene frequencies of the HPA-1 to HPA-13, Oe and Gov platelet antigen alleles in Taiwanese, Indonesian, Filipino and Thai populations. Int J Mol Med 2003;12:609-14.
26. Kickler TS, Herman JH, Furihata K, Kunicki TJ, Aster RH. Identification of Bakb, a new platelet specific antigen associated with post transfusion purpura. Blood 1988;71:894-8.
27. Okada N, Oda M, Sano T, Ito J, Shibata Y, Yamanaka M. Intracranial hemorrhage in utero due to fetomaternal Baka incompatibility. Acta Haematol Jpn 1988;51:1086-91.
28. Panzer S, Auerbach L, Cechova E, et al. Maternal alloimmunization against fetal platelet antigens: a prospective study. Br J Haematol 1995;90:655-60.
29. Shibata Y, Matsuda I, Miyaji T, Ichikawa Y. Yuk[a], a new platelet antigen system involved in two cases of neonatal alloimmune thrombocytopenia. Vox Sang 1986;50:177-80.
30. Bordin JO, Kelton JG, Warner MN, et al. Maternal immunization to Gov system alloantigens on human platelets. Transfusion 1997;37:823-8.

Pawinee Kupatawintu, BSc, National Blood Centre, Thai Red Cross Society, Henri Dunant Road, Pathumwan, Bangkok 10330, Thailand, and Department of Clinical Microscopy, Mahidol University, Bangkoknoi, Bangkok 10700, Thailand; Oytip Nathalang, PhD, Phramongkutklao College of Medicine, Rajthevi, Bangkok, Thailand; Rachanee O-Charoen, MD, National Blood Centre, Thai Red Cross Society, Bangkok, Thailand; and Pimpicha Patmasiriwat, PhD, Department of Clinical Microscopy, Mahidol University, Bangkok, Thailand.