DISTRIBUTION OF HUMAN PLATELET ANTIGENS IN THAI BLOOD DONORS USING SIMULTANEOUS PCR-SSP TECHNIQUE

PAWINEE KUPATAWINTU

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Pawinee Kupatawintu
.....
Miss. Pawinee Kupatawintu
Candidate

Oytip Nathalang
.....
Assoc. Prof. Oytip Nathalang, Ph.D.
Major-Advisor

R. O'Charoen
.....
Dr. Rachanee O-Charoen, M.D.
Co-Advisor

P. Patmasiriwat
.....
Assoc. Prof. Pimpicha Patmasiriwat, Ph.D.
Co-Advisor

Ramm Hoonsawat
.....
Assoc. Prof. Rassmidara Hoonsawat, Ph.D.
Dean
Faculty of Graduate Studies

Ratana Lawung
.....
Lect. Ratana Lawung, Ph.D.
Acting Chair
Master of Science Programme in
Medical Technology
Faculty of Medical Technology

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4 November, 2004

Miss. Pawinee Kupatawintu
Candidate

Assoc. Prof. Oytip Nathalang, Ph.D.
Chair

Dr. Rachanee O-Charoen, M.D.
Member

Assoc. Prof. Sasitorn Bejrachandra, M.D.
Member

Assoc.Prof.Pimpicha Patmasiriwat, Ph.D.
Member

Assoc. Prof. Rassmidara Hoonsawat, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Assoc. Prof. Chatchai Sornchai, M.D., M.Sc.
Dean
Faculty of Medical Technology
Mahidol University

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Pawinee Kupatawintu

DISTRIBUTION OF HUMAN PLATELET ANTIGENS IN THAI BLOOD DONORS USING SIMULTANEOUS PCR-SSP TECHNIQUE

PAWINEE KUPATAWINTU 4436679 MTMT/M

M.Sc.(MEDICAL TECHNOLOGY)

THESIS ADVISORS: OYTIP NATHALANG, PH.D., RACHANEE O-CHAROEN, M.D., PIMPICHA PATMASIRIWAT, PH.D.

ABSTRACT

Human platelet alloantigens (HPA) are important in fetomaternal alloimmune thrombocytopenia (FMAIT), post transfusion purpura (PTP), platelet transfusion refractoriness, passive alloimmune thrombocytopenia and transplantation-associated alloimmune thrombocytopenia**.** Thus, HPA genotyping is essential in patient diagnosis and treatment. In this study, HPA-1 to 6 and Gov alleles were analyzed using PCR with sequence specific primers (PCR-SSP) in 500 Thai blood donors, who have been HLA class I antigen-typed**.** It was found that 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% and 44.0%, respectively. In addition, for the Gov system, Gov^a and Gov^b showed frequencies of 49.1 % and 50.9%, respectively. The prevalence rates of HPA-1 to 6 and Gov gene frequencies (GFs) were consistent with those of other Asian populations rather than those of Caucasians. The results established a useful HPA and HLA-matched plateletpheresis donor file and provided an improvement in platelet alloantibody detection in alloimmune thrombocytopenic patients, and therefore, a more effective platelet transfusion program.

KEY WORDS: HUMAN PLATELET ANTIGEN (HPA)/ PCR-SSP/ THAIS/ GENE FREQUENCIES

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การกระจายของแอนติเจนเกล็ดเลือดในผู้บริจาคโลหิตคนไทย โดยการใช้วิธี พีซีอาร์ เอสเอสพี
(DISTRIBUTION OF HUMAN PLATELET ANTIGENS IN THAI BLOOD DONORS USING SIMULTANEOUS PCR-SSP TECHNIQUE)

ภาวิณี คุปตวินทุ 4436679 MTMT/M

วท.ม. (เทคนิคการแพทย์)

คณะกรรมการควบคุมวิทยานิพนธ์ : อ้อยทิพย์ ณ ถลาง, Ph.D., รัชนี โอเจริญ, พ.บ., พิมพิชฌา ปัทมสิริวัฒน์, Ph.D.

บทคัดย่อ

แอนติเจนของเกล็ดเลือด (human platelet alloantigens, HPA) มีความสำคัญในการเกิดปัญหา Fetomaternal alloimmune thrombocytopenia (FMAIT), post transfusion purpura (PTP), platelet transfusion refractoriness, passive alloimmune thrombocytopenia และ transplantation-associated alloimmune thrombocytopenia ดังนั้นการตรวจหาชนิดของแอนติเจนของเกล็ดเลือดจึงมีประโยชน์ในการวินิจฉัยโรครวมทั้งการรักษาโรค การศึกษาครั้งนี้ผู้วิจัยได้ทำการตรวจหาแอนติเจนของเกล็ดเลือด HPA-1 ถึง -6 และ Gov โดยใช้วิธีพีซีอาร์ เอสเอสพีในผู้บริจาคโลหิตคนไทยจำนวน 500 รายที่ได้ทำการตรวจหาแอนติเจนของ HLA Class I ไว้แล้ว

ผลการศึกษาพบว่าแอนติเจนที่พบได้ในผู้บริจาคทุกรายคือ HPA-4a ในขณะที่ HPA-1b, -2b, -5b และ -6b พบได้น้อยส่วน HPA-4b ไม่พบในการศึกษาครั้งนี้ Gene frequencies (GFs) ของ HPA-3a และ -3b พบได้ร้อยละ 56.0 และ 44.0 ตามลำดับ นอกจากนี้สำหรับแอนติเจนระบบ Gov พบว่า GFs ของ Gov^a และ Gov^b พบได้ร้อยละ 49.1 และ 50.9 ตามลำดับ เมื่อเปรียบเทียบ GFs ของ HPA-1 ถึง -6 และ Gov ในผู้บริจาคกับการศึกษาในกลุ่มประชากรอื่นพบว่า พบ GFs ใกล้เคียงกับคนเอเชียมากกว่าคนผิวขาว

จากการวิจัยครั้งนี้มีข้อสรุปได้ว่า การตรวจหาแอนติเจนของเกล็ดเลือดจะเป็นประโยชน์ในการคัดเลือกผู้บริจาคเกล็ดเลือดที่มี HPA และ HLA matched ให้กับผู้ป่วยและสามารถคัดเลือกเซลล์มาตรฐาน เพื่อนำมาใช้ในการตรวจสอบหาแอนติบอดีของเกล็ดเลือดในผู้ป่วยที่มีการสร้างภูมิต้านทานต่อเกล็ดเลือด อันจะมีผลทำให้การรักษาผู้ป่วยมีประสิทธิภาพดียิ่งขึ้น

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LIST OF ABBREVIATIONS

Abbreviations	Terms
α	Alpha
APC	Antigen Presenting Cell
β	Beta
BAC	Bacteria
^{o}C	Degree Celsius
CCI	Corrected Count Increment
CD	Cluster Differentiation
dH_2O	Distilled water
dNTPs	Deoxyriboneucleotide triphosphate
DNA	Deoxyribo-nucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-Link Immunosorbent
FITC	Fluorescein Isothiocyanate
FMAIT	Fetomaternal Alloimmune Thrombocytopenia
GF	Gene Frequency
GP	Glycoprotein
HDN	Hemolytic Disease of the Newborn
HGH	Human Growth Hormone
HGNC	Human Gene Nomenclature Committee
HLA	Human Leucocyte Antigen
HPA	Human Platelet Antigen
Ig	Immunoglobulin
iNOS	Inducible Nitric Oxide Synthase
L	Liter
ISBT	International Society of Blood Transfusion

LIST OF ABBREVIATIONS (CONT.)

Abbreviations	Terms
ISTH	International Society on Thrombosis and Haemostasis
kb	Kilo Base
KDa	Kilo Dalton
m	meter
MAIPA	Monoclonal Antibody Immobilization of Platelet Antigen
MHC	Major Histocompatibility Complex
µg	Microgram
µL	Microliter
µM	Micromole
min	Minute
mL	Millilitre
Mo-Ab	Monoclonal Antibody
NA	No Data Available
NET	Northeastern Thais
%	Percent
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR with Restriction Fragment Length Polymorphism
PCR-SSCP	PCR with Single Strand Conformation Polymorphism
PCR-SSO	PCR with Sequence Specific Oligonucleotide Probe
PCR-SSP	PCR with Sequence Specific Primers
PIFT	Platelet Immunofluorescence Test
PNC	Platelet Nomenclature Committee
PTP	Post Transfusion Purpura
PTR	Post Transfusion Refractoriness

LIST OF ABBREVIATIONS (CONT.)

Abbreviations	Terms
sec	Second
SNP	Single Nucleotide Polymorphism
SPRCA	Solid-Phase Red Cell Adherence Assay
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
UK	United Kingdom

CHAPTER I
INTRODUCTION

Platelet alloantigens are defined by alloantibodies directed against genetically determined structural differences in platelet membrane constituents. The antibodies are usually induced in individuals lacking an antigen upon exposure to antigen bearing platelets, mostly during pregnancy or transfusion (1). The first recognized and clinically most important alloantigen, human platelet antigen (HPA) -1a, was observed in 1959 by Van Longhem et al. in patients with the clinical picture of post transfusion purpura (2). The antibody enhanced platelet destruction, resulting in thrombocytopenia and hemorrhagic diathesis. Five clinical entities due to platelet specific alloantibodies can be distinguished: fetomaternal alloimmune thrombocytopenia (FMAIT), post transfusion purpura (PTP), platelet transfusion refractoriness (PTR), passive alloimmune thrombocytopenia and transplantation-associated alloimmune thrombocytopenia. FMAIT, PTP and PTR are three major problems caused by platelet alloantibodies (1, 3).

FMAIT is the platelet counterpart of red cell disorder and hemolytic disease of the newborn (HDN). It results from increased platelet destruction by maternal immunization against a fetal platelet antigen that is inherited from the father. Unlike HDN, FMAIT could occur during first pregnancy because platelet antigen appears in the fetus early in gestation and maternal antibodies can cross the placenta early in the 2^{nd} trimester, thereby inducing severe thrombocytopenia. The clinical picture presents as thrombocytopenia and skin and mucous bleeding. Intracranial hemorrhage may occur in utero usually between 30-35 weeks and occasionally before 20 weeks (4, 5). In Caucasian populations, FMAIT is observed in 1 of 2000-5000 newborns, about 75-90% of FMAIT cases are caused by HPA-1a antibodies, followed at a much lower frequency by HPA-5b (5-15%) and HPA-3a antibodies (2%), whereas in Asian populations, HPA-4b is found more frequently (1, 4, 6-9).

PTP is extremely rare and could occur 7-10 days after blood and blood component transfusion. The patients are sensitized by previous pregnancy or transfusion and respond to incompatible platelets by producing high titer of HPA antibodies. The exact mechanism by which the recipient's antigen negative platelets are also destroyed is not fully understood. Other platelet specific antibodies have been involved such as HPA-1b, -3a and -5a (10-13). Another possibility is that, it could occur by passive transfer of specific antiplatelet antibodies during blood or component transfusion (1, 14). The exact prevalence of PTP is not known; only 150 cases have been reported (14).

PTR, commonly found in multitransfused patients, is defined as low corrected count increment (CCI) after platelet transfusion. The 1-hour and 20-hour posttransfusion CCI were used to reflect the usefulness of platelets transfusion. The CCI of greater than 7.5 x 10^9/L/m^2 body surface area per number of platelets transfused at 1 hour or more than 4.5 at 20 hours is generally considered to be an adequate posttransfusion response. Immunological refractoriness to platelet transfusion is caused by alloantibodies reacting with transfused antigens (1, 15-17). Forty percent of patients are refractory due to human leukocyte antigen (HLA) immunization. Only 10-15% of HLA immunized patients additionally develop HPA antibodies. Conversely, in FMAIT and PTP, the most frequently observed are HPA antibodies (1, 16, 18, 19-21). The most common antibodies in PTR are HPA-5b, -1b, -5a, -2b, -3a, Gov^a and 1a, respectively (18, 20, 22). Previous studies in the Thai population have reported that the HLA and HPA antibodies were found in 49.6% and 6.1% of multitransfused patients. In addition, 40% of HPA antibodies could not be identified due to the low availability of HPA-genotyped panels (23, 24).

Up to 16 HPA systems are now designated, HPA-1 to -16w (25). Many reports have described the frequency of platelet alloantigens in different populations. Several remarkable differences are seen in gene frequencies of platelet antigens in different populations such as HPA-1, -4 and -5. In Asian populations, especially Japanese and Koreans, HPA-1b and -5b gene frequencies are extremely lower than Caucasians, whereas HPA-4b is higher than Caucasian populations (26, 27). Recent studies in Thai populations reported phenotype frequencies of HPA-1a, -2b, -3a, -4a and -4b among Northeastern Thais and Thai blood donors (28, 29).

Romphruk AV. et al. has reported the gene frequencies of HPA-1 to -6 systems from 19 provinces of Northeast Thailand and Shih MC. et al. has demonstrated the gene frequencies of HPA-1 to -13, Oe and Gov in 137 Thais who lived in Taiwan. It was found that the HPA-3a gene frequency (GF) was significantly higher than the previous Thai populations, which may be due to different population studies or different methods used (30, 31).

Up to now, more than 20 serological methods for the analysis of platelet antigens and antibodies have been reported, but only four techniques are suitable and reliable for routine testing, such as platelet immunofluorescence test (PIFT), monoclonal antibody immobilization of platelet antigen (MAIPA) assay, solid-phase red cell adherence assay (SPRCA) and ELISA-based techniques (26, 32). These methods are suitable for screening and identification of platelet antibodies. The limitations for platelet antigen typing by these methods are due to the availability of appropriate human typing sera because many human sera containing platelet specific alloantibodies also contain anti-HLA antibodies or other platelet reactive antibodies and these can cause unreliable results in tests, which utilize intact platelets. Only HPA-1a, a monoclonal antibody, which is made by conventional hybridoma techniques or phage display techniques, is described (27).

To compensate for this deficiency, HPA genotyping methods using the polymerase chain reaction (PCR), such as PCR with restriction fragment length polymorphism (PCR-RFLP), PCR with sequence specific oligonucleotide probe (PCR-SSO), PCR-single strand conformation polymorphism (PCR-SSCP) and PCR-sequence specific primers (PCR-SSP), which are determined by a single base-pair difference, have been introduced. PCR-RFLP is easy to perform and convenient for a small number of samples but incorrect assignments occur due to incomplete digestion. PCR-SSO is useful for large scale screening with one probe of HPA typing at the same time. In contrast, performing all platelet antigen typing needs multiple hybridization chambers and temperature control of the hybridization step, which is critical for obtaining good results. PCR-SSCP is useful for the identification of unknown variations of the allele, but there are various effects on the mobility of DNA such as temperature control and conditions of electrophoresis and the method need well-trained personnel. These PCR techniques are not suitable when rapid HPA genotyping

is required. The PCR-SSP has been shown to be a simpler and more reliable method for several HPA genotypes. This method can be performed in all HPA typing simultaneously, which is suitable for routine investigation. This method relies on the inability of Taq DNA polymerase to repair a single base mismatch at the 3' end of DNA primer. Thus, when the 3' nucleotide of a primer is complementary to the sequence at the site of allelic variation, the template will be amplified. The main disadvantage of this technique is that it needs a higher volume of DNA than other techniques (33, 34-36).

On the other hand, the screening and identification of platelet-specific antibodies requires testing serum against a panel of selected platelets with known HPA antigens. To be functional, the platelet panel must make it possible to identify with confidence those clinically significant alloantibodies that are most frequently encountered such as HPA-1, -4, -5 and Gov. The phenotypes of the platelet panel should be distributed such that each of the common alloantibodies, if it is the only one in a serum, can be clearly identified and most others at least tentatively excluded. The pattern of reactivity of a single alloantibody should not overlap with any other (37). A large scale of HPA genotyped donors would be helpful in selecting HPA homozygous platelet panels, which can be used for alloantibody identification by a variety of techniques, including PIFT, SPRCA, MAIPA and ELISA (38).

Severe FMAIT requires urgent treatment with compatible platelet concentrate. As prompt treatment is sometimes delayed owing to the unavailability of compatible platelet concentrate, patients with multiple transfusions, who have HPA and HLA antibodies, require large numbers of randomly donated platelets to be tested. Genotyped panels of HPA and HLA-typed donors could be accessed to effectively provide platelets for patients with alloantibodies. Additionally, thrombocytopenia is a major limited factor in any method used for platelet phenotyping. The HPA genotyping method is also useful for the diagnosis of various platelet disorders with severe thrombocytopenia, including FMAIT (4, 7, 24).

Objective:

The objective of this study is to introduce HPA genotyping using PCR-SSP in blood donors, who have been HLA class I antigen typed, and select HPA genotyped panels for investigation. Consequently, these results can provide HLA and HPA-matching data for patients with severe thrombocytopenia

CHAPTER II
LITERATURE REVIEW

There are several antigens on platelet cell surfaces, some of which are shared with other cell types, such as ABO, Le^a, Le^b, P, Cromer and HLA class I. The significant antibodies that react with platelets fall into 3 groups: ABO antibodies, HLA antibodies, and antibodies to platelet-specific antigens.

ABO antigens on platelets are intrinsic parts of platelet membranes and are also acquired by adsorption from plasma. The amount of ABO antigens present varies from individual to individual. ABO antibodies may cause the shortened survival of ABO-incompatible platelets and occasionally patients may become refractory on this basis, while there is no evidence of other red cell antibodies that affect platelet survival (37).

HLA class I antigens are cell-surface glycoproteins encoded by a series of closely linked genes on chromosome 6. These antigens are highly polymorphic and further categorized as A, B and C antigens. The amount of HLA-A or -B antigen is at least tenfold higher than that of HLA-C antigen, making it less necessary to match HLA-C locus antigens for donor-recipient compatibility in platelet transfusions. However, certain HLA-A or -B antigens also appear to be weakly expressed on platelets of some donors and may not elicit antibodies when mismatched. In general, the major cause of platelet refractoriness is involved with HLA class I antigens (39).

Non-HLA antibodies to antigenic structures on platelets are often termed “platelet specific”; these antigens may be found on other cells and tissues. Four platelet membrane glycoproteins have been found to be polymorphic and thus capable of inducing the production of alloimmune antibodies. Antibodies to platelet specific antigens are the major cause of FMAIT and PTP patients (25, 26, 40).

History of human platelet alloantigens

The platelet alloantigens were discovered in 1959; Van Longhem et al. reported the first platelet-specific alloantigen, Zw (2) and then, in 1961, Van Der Weerdt et al. reported the second alloantigen, Ko (41, 42). Shortly afterwards, anti-Pl^{a1} antibodies were detected and the specificity was identical to that of anti-Zw^{a} (42, 43). In 1980, Von Dem Borne et al. reported the third platelet alloantigen, Bak^{a}, using antiserum from the mother of a FMAIT patient (44). Shibata et al. identified the fourth platelet alloantigen, the Yuk^{a} / Yuk^{b}, in Japanese patients with FMAIT, 2 cases with Yuk^{a} in 1985 and one case with Yuk^{b} in 1986 (45, 46). Moreover, in 1985, Friedman & Aster reported a new maternal anti-platelet antibody; Pen^{a}, involved in FMAIT and congenital porencephaly cases, for which the specificity was identical to that of Yuk^{a} antisera (47). In 1989, Saji et al. identified the Sib^{a} antigen in a Japanese patient with PTR, which was similar to the Ko antigen (48).

In 1986, the fifth new alloantigen Br^{a}, was reported (49). This antigen was identified using the sera from FMAIT cases. New sixth and seventh alloantigens, Sr^{a} and Va^{a} were also found, both implicated in FMAIT (50, 51). In 1990, more than 10 alloantigens were reported using the serological techniques, almost all of them occurring at low frequency or private alloantigens involved in FMAIT cases; Ca^{a}, Tu^{a}, Mo^{a}, Max^{a}, La^{a}, Gro^{a}, Ly^{a}, Sit^{a}, Oe^{a}, and Pe^{a} (52-61). A unique platelet alloantigen, $Gov^{a/b}$, was reported by Kelton et al. in 1990. $Gov^{a/b}$ alloantibodies were detected in the sera of two multitransfused patients (62). Due to serological techniques, platelet specific alloantigens have been discovered because alloimmune thrombocytopenic patients were identified, which limited the discovery of other platelet specific alloantigens if an alloantibody against that antigen had not been reported.

Soon after the DNA sequence of the gene was determined by Fitzgerald et al. in 1987 (63), an amino acid polymorphism of Zw/Pl^{a} was discovered with a leucine/proline substitution at amino acid 33 by Newman et al (64). Later, Kuijper et al., Lyman et al., Wang et al. and Santoso et al. identified amino acid polymorphisms of Ko/Sib, Bak, Yuk/Pen and Br antigen systems, respectively (65-68). Recently, the

molecular basis of 16 platelet-specific antigens systems has been resolved and 15 of these are identified as a single nucleotide polymorphism (SNP), leading to single amino acid substitutions in the relevant membrane glycoprotein. Oe^a is an exception in that it is the result of an 'in frame' deletion of three nucleotides and that it has arisen from a mutation in Zw^b (Table 1) (25, 27). Their contributions made possible a number of useful clinical applications, including fetal testing and determination of the phenotype of several thrombocytopenic patients in the future.

Nomenclature of human platelet alloantigens

Historically, newly identified alloantigens have been assigned a name based on the patient from whom the alloantiserum was obtained. However, the same antigen could be discovered independently by multiple scientists and be given different names (for example, Zw and Pl^a, Ko and Sib etc.). To avoid confusion, the Platelet Nomenclature Committee (PNC) was created as a collaborative platform between the International Society of Blood Transfusion (ISBT) Platelet Working Party and International Society on Thrombosis and Haemostasis (ISTH) in order to formulate a new system nomenclature for platelet specific alloantigens (26). A platelet specific alloantigen is called a human platelet antigen (HPA) when its molecular basis has been defined. The different HPAs are grouped in systems based on having alloantibodies defining a given alloantigen and its antithetical alloantigen. HPA and their systems are numbered chronologically in order of the date of discovery. Allelic antigens will be designated alphabetically, in order of high to low frequency in the general population. A 'w' designation is added after the antigen name if an alloantibody against the antithetical antigen has not been reported. The list of HPA antigens in chronological order, including the original name and details of nucleotide and amino acid substitutions are shown in Table 1 (69).

Table 1 Human platelet antigens (HPA)

System	Antigen	Original names	Nucleotide change
HPA-1	HPA-1a	Zw^a, Pl^{a1}	176T > C
	HPA-1b	Zw^b, Pl^{a2}	
HPA-2	HPA-2a	Ko^b	482C > T
	HPA-2b	Ko^a, Sib^a	
HPA-3	HPA-3a	Bak^a, Lek^a	2621T > G
	HPA-3b	Bak^b	
HPA-4	HPA-4a	Yuk^b, Pen^a	506G > A
	HPA-4b	Yuk^a, Pen^b	
HPA-5	HPA-5a	Br^b, Zav^b	1600G > A
	HPA-5b	Br^a, Zav^a, Hc^a	
	HPA-6w	Ca^a, Tua	1544G > A
	HPA-7w	Mo^a	1297C > G
	HPA-8w	Sr^a	1984C > T
	HPA-9w	Max^a	2602G > A
	HPA-10w	La^a	263G > A
	HPA-11w	Gro^a	1976G > A
	HPA-12w	Ly^a	119G > A
	HPA-13w	Sit^a	2483C > T
	HPA-14w	Oe^a	1909_1911delAAG
HPA-15	HPA-15a	Gov^b	2108C > A
	HPA-15b	Gov^a	
	HPA-16bw	Duv^a	197C >T

Submissions all of HPAs should be made to the Secretary of the Committee. The PNC will take the following into account when considering their decision:

1. The genetic basis of the alloantigen must have been determined. Sequence data should be supplied, preferably genomic DNA sequence or a minimum of cDNA sequence.
2. The association between the genetic mutation and the reactivity of the alloantibodies with the allelic forms of the protein must been shown in protein-specific immunoassays.
3. At least two reference laboratories must have confirmed both the serological and molecular biology data.
4. The contributing laboratory should provide data from a population study of the index population. A pedigree with typed individuals from the index family and/or other typed families would be of additional value.
5. The contributing laboratory should make all reasonable efforts to make a blood sample available to one of the repository laboratories for establishment of a lymphoblastoid cell line.

HPA gene designation will use the terminology of the Gene Nomenclature Committee (HGNC), which relates to known functional aspects of the gene product. The symbol for a gene or gene cluster is italicized. Alleles are alternative forms of a gene and should be numbered by using the gene name, followed by an asterisk and number in italics. HPA genes and alleles are listed in Table 2 and 3. Platelet antigens without HPA assignment that have been published in peer-reviewed journals but for which the genetic basis has not yet been determined are designated by the first two or three letters of the surname of the patient who produced the first alloantibody. This abbreviation will be followed by a superscript ‘a’ or ‘b’, depending on whether the immune serum defines an alloantigen with a frequency >50% or <50% in the index population such as Va^a and Mou^a.

Table 2 HPA gene and reference sequences

Antigen	Gene	Ref_Seq
HPA-1, -4, -6w, -7w, -8w, -10w, -11w, -14w, -16w	*ITGB3*	NM_000212
HPA-2	*GP1BA*	NM_000173
HPA-3, -9w	*ITGA2B*	NM_000419
HPA-5, -13w	*ITGA2*	NM_002203
HPA-12w	*GP1BB*	NM_000407
HPA-15	*CD109*	NM_133493

Table 3 HPA alleles

HPA allele	Epitopes
*ITGB3*001*	HPA-1a HPA-4a
*ITGB3*002*	HPA-1b HPA-4a
*ITGB3*003*	HPA-1a HPA-4a HPA-10bw
*ITGB3*004*	HPA-1a HPA-4a HPA-16bw
*ITGB3*005*	HPA-1a HPA-4b
*ITGB3*006*	HPA-1a HPA-4a HPA-7bw
*ITGB3*007*	HPA-1a HPA-4a HPA-6bw
*ITGB3*008*	HPA-1b HPA-4a HPA-14bw
*ITGB3*009*	HPA-1a HPA-4a HPA-11bw
*ITGB3*010*	HPA-1a HPA4a HPA-8bw
*ITGA2B*001*	HPA-3a
*ITGA2B*002*	HPA-3b
*ITGA2B*003*	HPA-3b HPA-9bw
*ITGA2*001*	HPA-5a
*ITGA2*002*	HPA-5b
*ITGA2*003*	HPA-5a HPA-13bw
*GP1BA*001*	HPA-2a
*GP1BA*002*	HPA-2b

Table 3 HPA alleles (continued)

HPA allele	Epitopes
*GP1BB*001*	
*GP1BB*002*	HPA-12bw
*CD109*001*	HPA-15a
*CD109*002*	HPA-15b

Platelet glycoprotein polymorphisms

Inherited polymorphisms within platelet membrane glycoprotein (GP) genes can alter their antigenicity, regulate their expression levels, and modulate their functional properties. Among the variety of glycoproteins on human platelet surface, four contribute to immunogenic makeup to the platelet as follows (27, 40, 70, 71):

1. Glycoprotein IIb/IIIa complex (Intergrin $\alpha_{IIb}\beta_3$)
2. Glycoprotein Ia/IIa complex (Intergrin $\alpha_2\beta_1$)
3. Glycoprotein Ib/IX/V
4. Glycoprotein CD109

Polymorphisms of the glycoprotein IIb/IIIa complex

The GPIIb/IIIa complex is a heterodimeric intergrin consisting of noncovalently associated α and β subunits and mediates platelet aggregation via the binding of adhesive proteins, such as fibrinogen, fibronectin, vitronectin and von Willebrand factor (vWf) (Figure 1). Each of the first three molecules, GPIIb/IIIa, GPIa,IIa and GPIb/IX/V, recognized via the Arg-Gly-Asp (RGD) tripeptide. GPIIIa (CD61, β_3) is a glycosylated 90-kDa single protein consisting of three domains: 1) a large extracellular N-terminal region, which is highly cross-linked by 28 disulphide bonds, 2) a transmembrane domain and 3) a short cytoplasmic C-terminal segment. GPIIb (CD41, α_{IIb}) is synthesized as a single pro-GPIIb, which undergoes post-translational cleavage in the Golgi for cell surface expression as an extracellular 116-kDa heavy chain covalently associated by a single disulphide bond with the 22-kDa light chain, transmembrane protein. There are approximately 50,000-80,000 copies of

heterodimer per platelet. The gene coding for GPIIb/IIIa is located on the long arm of chromosome 17 within a single 260-kb segment. GPIIb is synthesized exclusively by megakaryocytes. Consequently, GPIIb/IIIa is a unique marker for platelets or cell lines with megakaryocytic phenotypes. Fourteen HPA antigens present on the GPIIb/IIIa complex (Table 4). The nucleotide variations were found on exon 2, 9, and 13 of the GPIIIa gene and on exon 26 of the GPIIb gene (69).

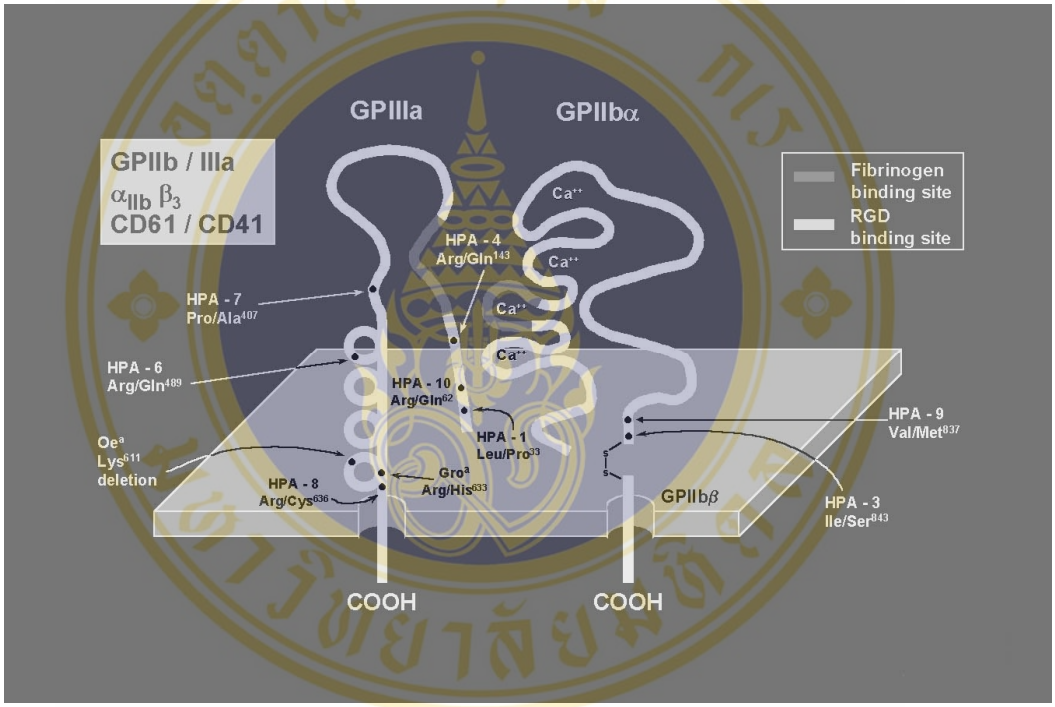

Figure 1 Glycoprotein IIb/IIa complex

Polymorphisms of glycoprotein Ia/IIa complex

The GPIa/IIa is another intergrin that contributes significantly to platelet function, and acts as the collagen receptor (Figure 2). This intergrin plays a fundamental role in the adhesion of blood platelets to both fibrillar and nonfibrillar collagens. GPIa (CD49b) is the 165-kDa α_2 chain and GPIIa (CD29) is the 145-kDa β_1 chain. There are approximately 800-2800 copies of GPIa/IIa per platelet. Three HPA antigens have been observed to be associated with variable expression between normal subjects and depend on the inheritance of at least three alleles of α_2 gene (Table 4).

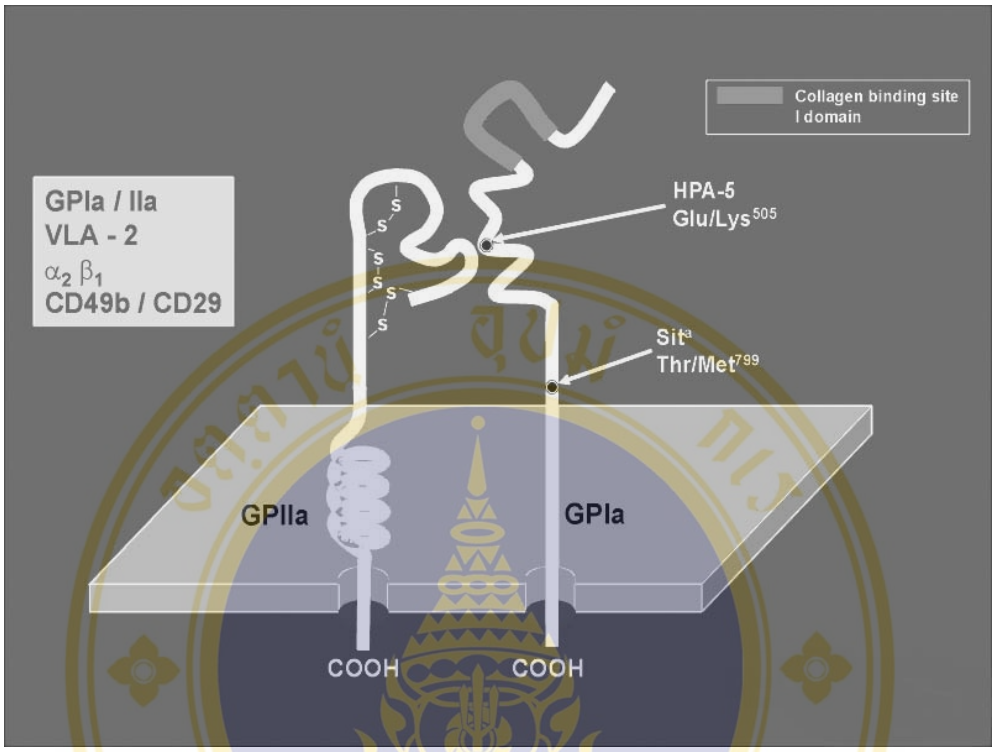

Figure 2 Glycoprotein Ia/IIa complex

Polymorphisms of GPIb/IX/V complex

The GPIb/IX/V complex is involved in the initial stages of platelet adhesion at high shear stress to damaged vessel walls via vWf in the subendothelial matrix. The pattern of membrane glycoproteins existing as functional complexes is repeated in the case of non-intergrin adhesion receptor complex Ib/IX/V. This receptor is composed of four transmembrane components: two molecules of GPIbα (CD42b, *Mr* 143 kDa) and GPIbβ (CD42c, *Mr* 22kDa), which are covalently linked by a single disulphide bond, and noncovalently associated with two molecules of GPIX (CD42a, *Mr* 20 kDa) and one molecule of GPV (CD42d, *Mr* 83 kDa) (Figure 3). There are approximately 25,000 copies of GPIb/IX and 12,000 copies of GPV per platelet and the whole complex is functionally associated with the low-affinity Fc receptor $Fc_{\gamma}RII$ (CD32). The primary vWf binding site has been localized within GPIbα but there is evidence that the other three components also contribute to receptor function. The GPIbα gene is on chromosome 17, the GPIbβ genes is on chromosome 22 and GPIX and GPV

genes are both on chromosome 3. The genetic basis has been determined for three HPA antigens present on this complex (Table 4).

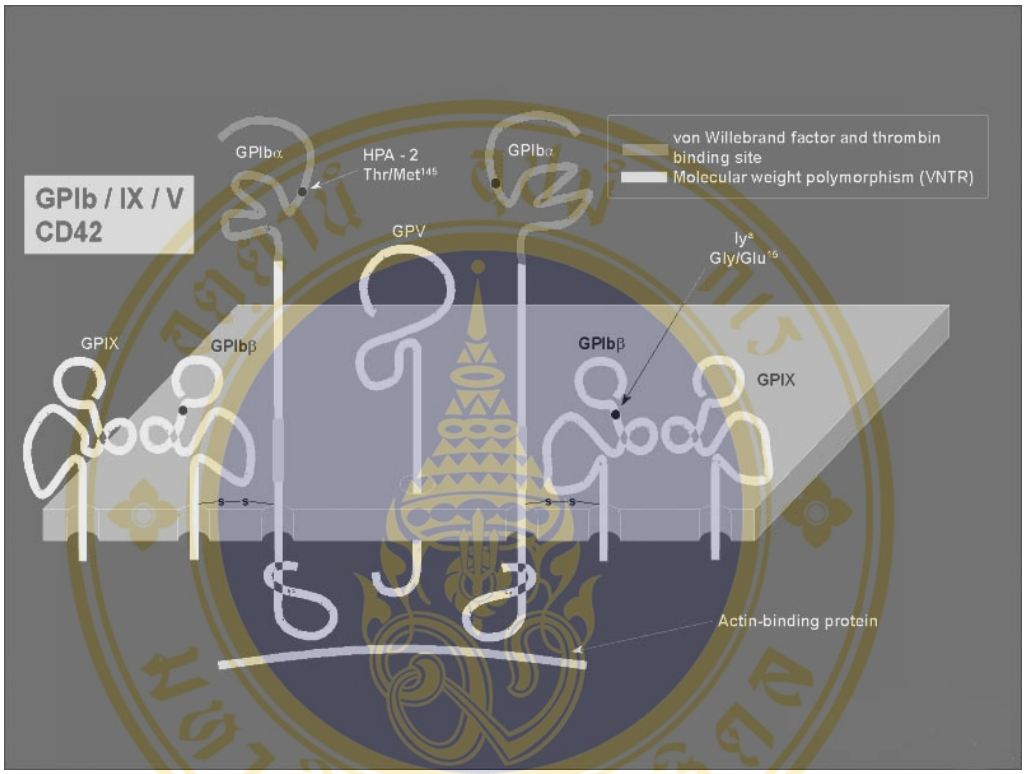

Figure 3 Glycoprotein Ib/IX/V complex

Polymorphisms of glycoprotein CD109

CD109 is the 175-kDa glycosylphosphatidylinositol (GPI)-linked glycoprotein found on platelets, monocytes, granulocytes, stimulated T-cells and CD34+ myeloid progenitor cells. CD109 expression on the platelet is low. There are approximately 2000 molecules per platelet. Although the precise function of CD109 remains obscure, this recent identification as a novel member of the α2M/C3, C4, C5 family of thioester-containing protease inhibitors and complement proteins suggests that it may be capable of mediating covalent cell-substrate and/or cell-cell interactions. The HPA-15 system is present on the CD109 molecule (Table 4).

Table 4 Platelet alloantigens present on glycoproteins and their cluster of differentiation (CD)

System	Antigen	Glycoprotein	CD
HPA-1	HPA-1a	GPIIIa	CD61
	HPA-1b		
HPA-2	HPA-2a	GPIbα	CD42b
	HPA-2b		
HPA-3	HPA-3a	GPIIb	CD41
	HPA-3b		
HPA-4	HPA-4a	GPIIIa	CD61
	HPA-4b		
HPA-5	HPA-5a	GPIa	CD49b
	HPA-5b		
	HPA-6w	GPIIIa	CD61
	HPA-7w	GPIIIa	CD61
	HPA-8w	GPIIIa	CD61
	HPA-9w	GPIIb	CD41
	HPA-10w	GPIIIa	CD61
	HPA-11w	GPIIIa	CD61
	HPA-12w	GPIbβ	CD42c
	HPA-13w	GPIa	CD49b
	HPA-14w	GPIIIa	CD61
HPA-15	HPA-15a	CD109	CD109
	HPA-15b		
	HPA-16bw	GPIIIa	CD61

Immune system for platelet alloimmunization

Two recipient T-cell recognition mechanisms have been shown to initiate alloimmunity. The direct pathway occurs when recipient T-helper cells directly interact with MHC class II molecules on donor antigen-presenting cells (APC), whereas the indirect pathway is analogous to the normal immune response. Indirect recognition occurs when allogeneic non-APC are administered to a recipient and is involved in the processing and presentation of allelic donor antigens (eg. MHC class I molecules) by recipient APC to recipient T-helper cells. The indirect pathway of allorecognition has been implicated in various transplant rejection models; the heart, kidney and skin (72-74). Within the context of indirect allorecognition, interactions between donor antigen and self-APC are critical to T-cell activation and subsequent antibody formation. In 1995, the animal models of platelet immunity demonstrated that allogeneic platelet stimulated IgG anti-donor immunity via indirect recognition. The indirect alloimmunity against platelets depends on the activation of inducible nitric oxide synthase (iNOS) within the recipient APC and could use either an endosomal or nonendosomal processing pathway to generate immunogenetic motifs that control the stimulation of IgG alloantibody formation (75).

HPA typing method

Over the last 25 years, many serological techniques have been developed for HPA typing. Only 3 techniques have become commonly used. First, the principle of the platelet immunofluorescence test (PIFT), is that platelet suspension antigen-antibody complex is detected by FITC anti-human IgG and examined under fluorescence microscope or flow cytometer. Second, the principle of the monoclonal antibody immobilization of platelet antigen (MAIPA) is that lysates containing the Mo-Ab platelets glycoprotein-antibodies immune complex are detected with a peroxidase-labelled antihuman IgG and substrate. The third test, the solid-phase red cell adherence assay (SPRCA), uses the principle that platelet monolayers antigen-

antibody complex are detected by anti-human IgG with anti-D sensitized red blood cells and examined by determination of the red blood cell agglutination pattern (26, 32).

To overcome the limitations of serological HPA phenotyping, especially involving thrombocytopenic patients and the lack of appropriate HPA antiserum, the molecular techniques have been introduced. Many SNP techniques have been applied to HPA genotyping. Only PCR-SSO, PCR-RFLP and PCR-SSP are widely used. The principle of PCR-SSO is that hybridization products of amplified DNAs that are attached to a nylon membrane support with labeled allele-specific reporter oligonucleotide probe are detected by chromogenic substrate such as avidin-horseradish peroxidase. In PCR-RFLP, the principle is that PCR products of restriction enzyme digestion are electrophoresed on polyacrylamide or agarose gel and the DNA fragmentation pattern is revealed by UV light. In PCR-SSP, the principle is that the amplified products, which are complementary to the sequence at the 3' nucleotide of the primer, are examined by agarose gel electrophoresis. According to genotyped technology development, new techniques are introduced to HPA genotyping such as differential melting curve profile analysis (LightCyclerTM) and 5'-Nuclease assay (TaqManTM assay). Both techniques are not suitable for the laboratory with a limited budget and need well trained staff. PCR-SSP is currently the most widely used technique for HPA genotyping because it is a relatively simply procedure, a more reliable method and suitable for routine investigation (25, 33).

CHAPTER III
MATERIALS AND METHODS

Materials

1. Subjects

Five-hundred blood samples from non-repeated healthy Thai blood donors at the National Blood Centre, Thai Red Cross Society, who had been HLA class I (HLA-A and -B) antigen-typed were collected for HPA-1 to -6 and Gov genotyping. Informed consent was obtained from all subjects.

2. Known HPA-1 to -6 and Gov

Twenty-six DNA samples provided by the Australian Platelet Antibody Workshop in association with the Australian and New Zealand Society of Blood Transfusion and 12th International Society of Blood Transfusion Platelet Immunology Workshop in association with the International Society of Blood Transfusion were used as the standard in PCR-SSP genotyping method.

Methods

1. Blood Collection

Three millilitres of EDTA blood samples were collected from blood donors at the end of blood donation step. The samples were kept at 4^{o}C for storage up to 1 week.

2. DNA extraction

Genomic DNA was extracted from whole blood of the subjects by the salting out technique (Pel-Freez, Brown Deer, WI, USA). The method used follows:

2.1 Add 1 mL of whole blood to the 1.5 mL polypropylene tube. Centrifuge for 1 min at 10,000 x *g*.

2.2 Remove plasma supernatant. Add 1 mL cold red cell lysis buffer, vortex for 30 sec. Centrifuge for 1 min at 10,000 x *g*.

2.3 Decant hemolyzed supernatant. Add 1 mL cold red cell lysis buffer, vortex for 5 sec. Centrifuge for 1 min at 10,000 x *g*.

2.4 Decant supernatant. Add 125 µL of protease to pellet, vortex for 5 sec. Incubate at 65°C for 10 min. Vortex every 2-3 min.

2.5 Add 275 µL protein clearing solution, vortex for 5 sec. Incubate on ice for 10 min. Centrifuge for 5 min at 12,000 x *g*.

2.6 Carefully transfer supernatant to a clean 1.5 ml polypropylene tube. Slowly add 500 µL of cold 90% ethanol. Precipitate DNA by inverting tube 6-10 times.

2.7 Centrifuge DNA pellet at 12,000 x *g* for 5 min.

2.8 Decant supernatant. Add 1 mL of 70% ethanol, vortex for 5 sec and centrifuge for 2 min at 12,000 x g.

2.9 Remove ethanol by decanting, air dry the DNA pellet. Add 150 µL of DNA suspension buffer. Incubate for 15 min at 65°C. Vortex gently to resuspend.

2.10 Measure DNA concentration by UV spectrophotometer at wavelength 260/280 nm.

2.11 Adjust all DNA at a concentration 100-200 ng/µL by DNA suspension buffer and keep at -30°C until performing PCR-SSP.

3. HPA-1 to -6 and Gov primers

3.1 The HPA-1 to -6 primers were selected from Cavanagh G. et al. and Skogen B. et al. with some modifications (33, 34, 38). Gov primers were selected from Schuh A.C. et al. HPA-3b allele-specific antisense primer was modified by extending 2 more bases for increasing HPA-3b genotyping specificity. The primer concentrations were optimized in the same PCR condition by adjusting competitive reactivity between allele specific primers and control primers (Human Growth Hormone gene; HGH) at the concentration from 0.1 µM to 0.5 µM.

3.2 The primers and primer concentration used in this study are listed in Table 5.

Table 5 Sequence of the primers for HPA-1 to 6, Gov and HGH internal control

Primer	Sequence	Final Conc. (μM)
HPA-1a	5′ TCA CAG CGA GGT GAG GCC A 3′	
HPA-1b	5′ TCA CAG CGA GGT GAG GCC G 3′	0.1
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′	0.1
HPA-2 common	5′ TCA GCA TTG TCC TGC AGC CA 3′	
HPA-3a	5′ TGG ACT GGG GGC TGC CCA T 3′	
HPA-3b	5′ GGT GGA CTG GGG GCT GCC CAG 3′	0.2
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′	0.1
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′	0.5
HPA-5 common	5′ CTC TCA TGG AAA ATG GCA GTA 3′	
HPA-6a	5′ GAC GAG TGC AGC CCC CG 3′	
HPA-6b	5′ GGA CGA GTG CAG CCC CCA 3′	0.2
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′	0.4
Gov common	5′ ATG ACC TTA TGA TGA CCT ATT C 3′	
HGH control 1	5′ GCC TTC CCA ACC ATT CCC TTA 3′	0.4 (HPA-1-4 & HPA-6) 0.3(HPA-3b) 0.1 (HPA-5) 0.2 (Gov)
HGH control 2	5′ TCA CGG ATT TCT GTT GTG TTT C 3′	

3.3 The primer mix contains the specific primer, the common primer and 2 control primers. To make the working primer mix to be 2x final concentration, an equal volume of 8x final concentration of each primer was added in to the 1.5 mL microcentrifuge tube. The concentration of working primers mixes are shown in Table 6.

Table 6 Concentration of each HPA genotyping stock primer mix

HPA genotyping working primer mix	Specific primer (μM)	Common primer (μM)	Control primers 1 (μM)	Control primers 2 (μM)
HPA-1a	0.8	0.8	3.2	3.2
HPA-1b	0.8	0.8	3.2	3.2
HPA-2a	0.8	0.8	3.2	3.2
HPA-2b	0.8	0.8	3.2	3.2
HPA-3a	1.6	1.6	2.4	2.4
HPA-3b	1.6	1.6	3.2	3.2
HPA-4a	0.8	0.8	3.2	3.2
HPA-4b	0.8	0.8	3.2	3.2
HPA-5a	4.0	4.0	0.8	0.8
HPA-5b	4.0	4.0	0.8	0.8
HPA-6a	1.6	1.6	3.2	3.2
HPA-6b	1.6	1.6	3.2	3.2
Gov[a]	3.2	3.2	1.6	1.6
Gov[b]	3.2	3.2	1.6	1.6

3.4 Aliquot 5 μL of each stock primer was mixed into 0.2 thin walled tube in strips. One drop of Chill-out 14™ liquid wax was added (MJ Research, Waltham, MA, USA Waltham, MA, USA). The stock primer mix could be kept at -30°C until use.

4. HPA-1 to -6 and Gov PCR-SSP genotyping

The optimal DNA template and *Taq* DNA polymerase (Promega, Madison, WI USA) were adjusted by testing the reactivity of known HPA-1 to -6 and Gov with the same PCR condition. The DNA template was adjusted at a concentration from 0.006-0.200 μg/μL. The optimal DNA concentration was from 0.100-0.200 μg/μL and the minimum DNA template concentration was 0.050 μg/μL. *Taq* DNA polymerase was adjusted from 0.3-1 units/reaction. The optimal *Taq* DNA polymerase was 0.75 units/reaction. The method used follows:

4.1 For each sample to be tested, thaw 2 strips of 14 tubes each containing 5 μL of various HPA-1 to -6 and Gov primer mixes.

4.2 Make a master mix of all 14 (+2) reactions as follows:

dH2O	28.8	μL
10x reaction buffer	16.0	μL
dNTPs mix (2.5 mM each)	3.2	μL
Taq polymerase (5 units/μL)	2.4	μL
DNA (approx 100 ng/μL)	32.0	μL
Total volume	82.4	μL

4.3 Add 5 μL of master mix to each of the 14 tubes of stock primer mix.

4.4 Amplify 10 μL aliquots mixture in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) or PTC-200 (MJ Research, Waltham, MA, USA).

The cycle parameters of the PCR program were similar to Cavanagh G. et al. and Metcalfe P. et al. (34, 76). The PCR program was:

One cycle	96°C for 1 min
Five cycles	96°C for 25 sec
	68°C for 45 sec
	72°C for 30 sec
Twenty-eight cycles	96°C for 25 sec
	61°C for 45 sec
	72°C for 30 sec
One cycle	72°C for 3 min

5. Interpretation of PCR-SSP

The DNA amplicon were electrophoresed through 2.0% agarose gel containing 0.5 µg/mL ethidium bromide. The gel was run at 100 V for 30 min in 0.5X TBE and visualized under UV transilluminator. The reaction was photographed and the HPA alleles were assessed.

6. Reproducibility of HPA-1 to -6 and Gov genotyping

Ten samples were randomly repeated for HPA-1 to -6 and Gov genotyping by PCR-SSP to test reproducibility.

7. 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 x *af*[a] x *af*[b] x N for heterozygotes and *af*[a or b]^2 x 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 Fisher's exact test (77).

CHAPTER IV
RESULTS

HPA-1 to -6 and Gov PCR-SSP genotyping

1. Interpretation of PCR-SSP

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 for Gov, respectively. In all reactions, the human growth hormone (HGH) internal control gave an expected band of 429 bp (Table 7).

Table 7 Product sizes of HPA-1 to -6, Gov and HGH internal control obtained by the PCR-SSP

HPA	Product size (bp)	HPA	Product size (bp)
HPA-1a		HPA-5a	
HPA-1b	90	HPA-5b	246
HPA-2a		HPA-6a	238
HPA-2b	258	HPA-6b	239
HPA-3a	267	Gov[a]	
HPA-3b	269	Gov[b]	225
HPA-4a		HGH control	
HPA-4b	120	HGH control	429

Additional smaller, nonspecific products were seen in HPA-3b and -6b, while a larger nonspecific product was also seen in HPA-4b, but these did not interfere with the interpretation of the HPA genotypes (Fig. 4).

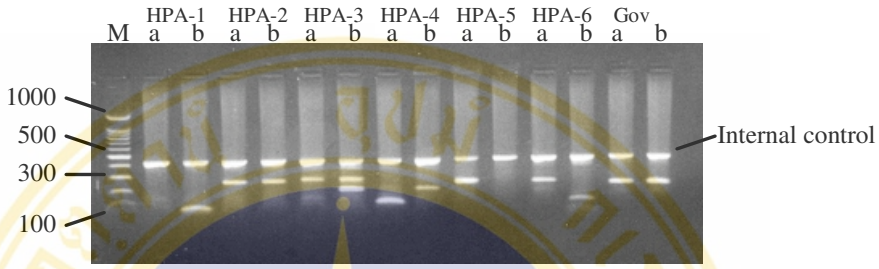

Figure 4 A representative case for simultaneous HPA-1 to 6 and Gov genotyping by PCR-SSP. The 429-bp amplification product of the HGH control primers is present in all lanes, which shows that amplification has occurred optimally. The genotype was deduced from the presence or absence of amplification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

2. Sensitivity of the PCR-SSP

The DNA template was diluted by DNA suspension buffer (Pel-Freez, WI, USA) from the concentration of 0.006 to 0.200 μg/μL. The PCR-SSP was performed using different concentrations of the DNA template. It was found that the internal control band can be demonstrated in all HPA-1 to -6 and Gov when the DNA concentrations was 0.006 μg/μL, whereas only the specific bands of HPA-4a, -4b, Gov^{a} and Gov^{b} were weakly detected. At the DNA concentration of 0.012 and 0.025 μg/μL, the specific band of HPA-5b was not clearly detected. Starting from DNA concentration of 0.05 μg/μL, the results of HPA-1 to -6 and Gov genotyping were clearly identified (Table 8).

Table 8 Selection of optimum dilution of DNA template for HPA genotyping by PCR-SSP

HPA	Band	DNA Concentration (μg/μL)					
		0.006	**0.012**	**0.025**	**0.050**	**0.100**	**0.200**
HPA-1a	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-1b	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-2a	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-2b	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-3a	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-3b	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-4a	Control	+	+	+	+	+	+
	Specific	w+	+	+	+	+	+
HPA-4b	Control	+	+	+	+	+	+
	Specific	w+	+	+	+	+	+
HPA-5a	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-5b	Control	+	+	+	+	+	+
	Specific	-	w+	w+	+	+	+
HPA-6a	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
HPA-6b	Control	+	+	+	+	+	+
	Specific	-	+	+	+	+	+
Gov[a]	Control	+	+	+	+	+	+
	Specific	w+	+	+	+	+	+
Gov[b]	Control	+	+	+	+	+	+
	Specific	w+	+	+	+	+	+

+ = positive band, w+ = weak positive band, - = negative band

3. Reproducibility of HPA-1 to -6 and Gov genotyping

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 the first round of testing. Moreover, this PCR-SSP of HPA typing method was validated using 26 HPA reference materials typed by alternative techniques and compared with other laboratories. The results showed a 100% concordance. The results of HPA-1 to 6 and Gov typing are shown in Table 9.

Table 9 HPA-1 to 6 and Gov typing results of 26 reference standard samples

Sample No.	HPA													
	-1a	**-1b**	**-2a**	**-2b**	**-3a**	**-3b**	**-4a**	**-4b**	**-5a**	**-5b**	**-6a**	**-6b**	**Gov[a]**	**Gov[b]**
2001A	+	0	+	0	+	+	+	0	0	+				
2001B	+	+	+	0	0	+	+	0	+	+				
2001C	+	0	+	0	0	+	+	+	+	0				
2001D	0	+	+	0	+	+	+	0	0	+				
2002A	+	0	+	0	0	+	+	0	+	0	+	0		
2002B	+	0	+	0	0	+	+	+	+	0	+	0		
2002C	+	+	+	+	0	+	+	0	+	0	+	0		
2002D	0	+	+	0	+	+	+	0	+	+	+	0		
2003A	+	0	+	0	0	+	+	+	+	0	+	0	+	+
2003B	0	+	+	0	+	0	+	0	+	+	+	0	+	0
2003C	+	0	+	0	+	0	+	0	0	+	+	0	+	0
2003D	0	+	+	+	+	+	+	0	+	0	+	0	+	+
2004-1	+	+	+	0	+	+	+	0	0	+	+	0	+	0
2004-2	+	+	0	+	+	+	+	0	+	0	+	0	+	+
2004-3	+	+	+	+	+	+	+	0	+	+	+	0	0	+
2004-4	+	+	+	0	+	+	+	0	+	+	+	0	0	+
2004-5	+	0	+	+	+	+	+	0	+	0	+	0	+	+
2004-6	+	+	+	0	0	+	+	0	+	0	+	0	+	0
2004-7	+	+	+	+	0	+	+	0	+	0	+	+	+	+

Table 9 (continued) HPA-1 to 6 and Gov typing results of 26 reference standard samples

Sample No.	HPA													
	-1a	**-1b**	**-2a**	**-2b**	**-3a**	**-3b**	**-4a**	**-4b**	**-5a**	**-5b**	**-6a**	**-6b**	**Gov[a]**	**Gov[b]**
2004-8	0	+	+	0	0	+	+	0	+	0	+	0	+	+
2004-9	+	0	0	+	+	0	+	0	+	0	+	0	0	+
2004-10	+	0	+	0	+	0	+	0	0	+	+	0	+	+
2004A	+	+	0	+	+	+	+	0	+	0	+	0	+	+
2004B	0	+	+	0	+	0	+	0	+	+	+	0	+	+
2004C	+	0	+	0	0	+	+	+	+	0	+	0	+	+
2004D	+	+	+	0	0	+	+	0	+	+	+	0	0	+

HPA-1 to -6 and Gov gene frequencies

Five hundred non-repeated Thai blood donors, 369 males and 131 females with ages ranging from 19 to 58 years, were typed HPA-1 to -6 and Gov antigens. The sample comprised 113 in group A, 134 in group B, 43 in group AB and 210 in group O donors. The genotype and gene frequencies are shown in Table 10. 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% and 44.0%, respectively. In addition, for the Gov system, Gov^a and Gov^b showed frequencies of 49.1 % and 50.9%, respectively.

Table 10 The genotype and gene frequencies of HPA-1 to -6 and Gov in 500 Thai blood donors

Genotype	No.	Genotype frequencies (%)	Gene frequencies	
			Gene	(%)
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		

Comparison of HPA-1 to -6 and Gov gene frequencies

A comparison of HPA-1 to 6 and Gov gene frequencies among Asian and Caucasian studies is presented in Tables 11-13 (22, 30, 78-86). The HPA-1 to 6 frequencies in Thai blood donors are more similar to Northeastern Thais (NET) and those of other Asians rather than those of Caucasians. The Gov[a] and Gov[b] frequencies in our study showed similar results to previous studies (31, 86) but were significantly different from the study in UK populations ($p<0.01$) (22).

Table 11 Gene frequencies of HPA-1 to 6 in 500 Thai blood donors and Asian populations

HPA	Thailand N=500	NET N=300 (30)	Taiwan N=300 (78)	Hong Kong N=100 (79)	Korea N=100 (80)	Japan N=331 (81, 82)
-1a	98.5	97.2	*99.7*[a]	99.5	99.5	*99.8*[b]
-1b	1.5	2.8	*0.3*[a]	0.5	0.5	*0.2*[b]
-2a	95.2	93.8	96.0	97.5	*87.0*[c]	*89.8*[c]
-2b	4.8	6.2	4.0	2.5	*13.0*[c]	*10.2*[c]
-3a	56.0	53.3	57.5	52.5	*67.0*[b]	59.4
-3b	44.0	46.7	42.5	47.5	*33.0*[b]	40.6
-4a	100.0	100.0	99.8	100.0	100.0	*98.9*[b]
-4b	0.0	0.0	0.2	0.0	0.0	*1.1*[b]
-5a	96.8	96.3	*98.5*[a]	96.5	97.0	96.0
-5b	3.2	3.7	*1.5*[a]	3.5	3.0	4.0
-6a	98.6	98.5	*96.3*[b]	NA	NA	97.3
-6b	1.4	1.5	*3.7*[b]	NA	NA	2.7

N = Number tested

Thai: Thai blood donors, NET: North-eastern Thais, NA: No data available;

[a]$P< 0.05$ [b]$P< 0.01$ [c] $P< 0.001$

Table 12 Gene frequencies of HPA-1 to 6 in 500 Thai blood donors and Caucasian populations

HPA	Thailand N=500	Spain N=107 (83)	Wales N=392 (84)	Australia N=1,000 (85)	Norway N=105 (86)
-1a	98.5	*74.8*[c]	*82.5*[c]	*85.8*[c]	*86.7*[c]
-1b	1.5	*25.2*[c]	*17.5*[c]	*14.2*[c]	*13.3*[c]
-2a	95.2	*81.8*[c]	*90.9*[c]	*92.7*[b]	94.3
-2b	4.8	*18.2*[c]	*9.1*[c]	*7.3*[b]	5.7
-3a	56.0	*68.2*[b]	*60.7*[a]	*61.9*[b]	*47.1*[a]
-3b	44.0	*31.8*[b]	*39.3*[a]	*38.1*[b]	*52.9*[a]
-4a	100.0	100.0	100.0	100.0	100.0
-4b	0.0	0.0	0.0	0.0	0.0
-5a	96.8	*86.1*[c]	*90.3*[c]	*90.5*[c]	*92.9*[b]
-5b	3.2	*13.9*[c]	*9.7*[c]	*9.5*[c]	*7.1*[b]
-6a	98.6	100.0	*100.0*[a]	NA	NA
-6a	1.4	0.0	*0.0*[a]	NA	NA

N = Number tested

Thai: Thai blood donors, NET: North-eastern Thais, NA: No data available;

[a]P< 0.05 [b]P< 0.01 [c] P< 0.001

Table 13 Gene frequencies of Gov in 500 Thai blood donors and different populations

HPA	Thailand[a] N=500	Norway N=105 (86)	UK N=113 (22)	Taiwan N=566 (31)	Indonesia N=107 (31)	Thailand N=137 (31)	Philippines N=100 (31)
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

N = Number tested

[a]Thai: Thai blood donors; [b]P< 0.01

CHAPTER V
DISCUSSION

The investigation of HPA gene and genotype frequencies is important in both population studies and clinical transfusion practice where HPA-typed platelets may be required for alloimmunized patients. This is due to the fact that the failure rate of 10-32% of platelet count increment has been observed in HLA-match platelet transfusion. Other immunological investigations, which include platelet antibody testing to identify HPA specificity, should be performed. Generally, platelet specific antibodies have been recognized most frequently in patients with broad spectrum HLA antibodies (1, 15, 16, 18, 19). These patients have beneficial results from HLA- and HPA-matched platelet transfusion support. Thus, providing more data in 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 product (38).

Recent studies reported gene frequencies of HPA-1 to 13, Oe and Gov alleles in Taiwanese, Indonesian, Filipino and Thai populations using the PCR-RFLP technique (87). Although the results were consistent with other previous findings in Asian populations (78-81), the PCR-RFLP technique has some disadvantages such as being time consuming and labour intensive and causing misinterpretation due to incomplete enzyme digestion of the amplicon (33, 38). In alloimmune thrombocytopenia, especially in FMAIT investigation requires prompt results for antibody identification as well as provides HLA- or HPA-matched platelet transfusion (12, 24, 87). The HPA genotyping technique, which is rapid and able to detect all of clinical significant HPA antigens at the same time will be useful for routine investigations. The PCR-SSP has been widely used as a method for simultaneous genotyping of multiple HPAs and has replaced other DNA-based HPA

typing methods in clinical laboratory testing (33-36, 38, 78, 83-85). The whole process of PCR-SSP can be completed within 4 hours, starting with genomic DNA extraction from blood to the results of all HPA genotypes. Moreover, in this study, the PCR-SSP technique gives good results its sensitivity, reliability and reproducibility. Because the HPA antibodies, commonly found in alloimmunized patient were HPA-1a, -1b, -2b, -3a, -3b, -4a, -4b, -5a, -5b, and -6b in Caucasian and Asian populations, the HPA-1 to -6 genotyping have been implemented for routine testing (1, 4, 7, 8-10, 14, 18-20). Recently, the Gov antibodies have been reported for their clinical importance especially in FMAIT, PTP and platelet refractoriness. Although the incidence of Gov antibodies was lower than the HPA-1 system, the incidence was equal to HPA-5 system antibodies in Caucasian populations (22). 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. Although we found non specific products in HPA-3b, -6b and -4b, which were identified as human DNA sequence from the BAC clone on other chromosomes by DNA sequencing, these products did not interfere with the interpretation of the HPA genotypes. This HPA genotyping method is suitable in platelet antigen genotyping for either alloimmune thrombocytopenia or in genetic populations.

Recent studies reported HPA genotype frequencies in different populations, in order to predict the risk of developing platelet-specific alloantibodies, which depends on the difference among HPA frequencies. High frequency antigens could result in a higher risk than low frequency antigens (78, 80, 81, 85). From this study, the distributions of HPA-1 to -6 genotypes in Thai blood donors living in Bangkok are consistent with those found in north-eastern Thailand (30). Concerning the genotype frequencies of HPA-1 to -6 and Gov from 500 Thai blood donors, there is no risk of developing HPA-1a, -2a, -4a, -5a and -6a antibodies in the Thai population because HPA-1b1b, -2b2b, -4b4b, -5b5b and -6b6b were not found, which is similar to other studies in Asian populations (30, 31, 78-81). In contrast, the genotype frequencies of HPA-1b1b were 2-4% in Caucasian populations (83-86). In FMAIT cases, it was found that 75-90% caused by HPA-1a antibodies (4, 7). Moreover, FMAIT caused by HPA-4b antibodies were reported in Japanese populations (8). This is because the genotype frequency of HPA-4b is significant higher than Thais and other Asians (81).

Additionally, HPA antibodies due to low frequency antigens such as HPA -1b, -2b, -3a, -3b, -4b, -5b, -6b, Gov^{a} and Gov^{b} could be found in both Asian and Caucasian populations. Antibodies to HPA-1b, -3a, -5b and Gov^{a} are commonly found in FMAIT cases, while, HPA-1b, -2b, -3a, -5b and Gov^{a} are commonly found in PTR (8, 9, 18). Although the specificities of HPA antibodies to these low frequency antigens are similar in Asian and Caucasian populations, previous studies have shown that incidence of HPA alloimmunization of FMAIT (0.6%) and PTR (6%) cases in Thai populations were lower than Caucasian populations (6% in FMAIT and 8% in PTR) (9, 18, 23). This may be due to the fact that the frequencies of those HPA antigens in Caucasians populations are significantly higher than in Asian populations (30, 78-86).

The ability to identify platelet-specific antibodies depends on not only the technique used but also having appropriate HPA panel cells. To be functional, an HPA panel must make it possible to identify with confidence those clinically significant alloantibodies that are most frequently found. The pattern of reactivity for most common single alloantibodies should not overlap with other antigens (37). Due to the unavailablity of commercial HPA panels, all laboratories need to find their own HPA panels for routine investigation. From this study, a new HPA panel was selected from 210 group O, HPA typed donors. HPA-1b, -2a, -3b, -5a, -6a, -6b, Gov^{a} and Gov^{b} have been newly added to these panels. All panels have been completely typed for HPA-1 to -6 and Gov. The limitations of these new HPA panels are first, the ability to identify HPA-1a, -2a, -4a, -5a and -6a, second, the ability to confirm HPA-1b and -6b antibodies and third, the ability to detect HPA-4b antibodies. Further HPA typed in group O donors for HPA -1a, -4a, -4b, and -5a will be helpful in HPA panel development in the future. Furthermore, the data from this study could provide more HPA matched platelet donors to support alloimmune thrombocytopenic patients who need HPA compatible platelet transfusion.

CHAPTER VI
CONCLUSION

This study showed that the distributions of HPA-1 to -6 genotypes in Thai blood donors living in Bangkok are consistent with those found in north-eastern Thailand. However, it is recommended that further population studies be conducted comparing other subgroups, such as northern and southern Thai populations.

From this study, a new HPA panel was selected but a few limitations still existed. Further HPA typed in group O donors for HPA -1a, -4a, -4b, and -5a will be helpful in HPA panel development in the future, to increase the utility of panel selection.

In alloimmune thrombocytopenia, especially in FMAIT investigation requires prompt results for antibody identification as well as provides HLA- or HPA-matched platelet transfusion. The HPA genotyping technique, which is rapid and able to detect all of the clinically significant HPA antigens at the same time will be useful for routine investigations. The implementation of the HPA genotyping system using the PCR-SSP technique in both donors and patients is beneficial in platelet transfusion therapy in order to provide HPA-matched platelet donors and increase the capability for platelet alloantibody investigation.

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APPENDIX

Appendix A

Reagent preparation

1. **Calculation of neat stock primer concentration**

   The concentration in μM is calculated using the following formula:

   Concentration (μM) = B/A

   B (μg/L) = (μg/OD x OD purified) x 1000 mL

   A = molecular weight of neat stock primer

2. **20 μM stock primer**

   20 μM stock primer is made up to 400 μL by using the following calculation:

   Neat stock primer (μL) = (400 μL x 20 μM) / neat stock primer (μM)

   Make up to 400 μL by distilled water

   20 μM stock primers are stored at -30^{o}C

3. **Working primer**

   Working primer is made up to 1 mL from 20 μM stock primer by using the following calculation:

   Stock primer (μL) = (500 μL x working primer (μM)) / 20 μM)

   Make up to 500 μL by distilled water

   Working primers are stored at -30^{o}C

4. 10X reaction buffer

$(NH_4)_2SO_4$	2.114	g
1M tris HCl pH 8.8	67	mL
Tween 20	0.1	mL
1M $MgCl_2$	1.5	mL

Make up to 100 mL with distilled water

5. 1M tris HCl

Tris HCl	15.76	g

Make up to 100 mL with distilled water

Adjust pH 8.8 with NaOH

6. 2% agarose

LE agarose	0.8	g

Make up to 40 mL with 0.5X TBE buffer

Melt by microwave

7. 0.5X TBE

Tris Borate EDTA	8.5	g

Make up to 1000 mL with distilled water

8. Loading buffer

Orange G	50	mg
Ficoll	2	g
0.5M EDTA	2	mL

Make up to 20 mL with distilled water

9. 0.5M EDTA

EDTA	37.2	g

Make up to 100 mL with distilled water

Appendix B

Chemicals and Reagents

1.	DNA isolation kit	Pel-Freez	USA
2.	Ethanol (99.9%)	Merck	Germany
3.	HPA primer	BSU	Thailand
4.	Chill-out 14™ liquid wax	MJ Research	USA
5.	*Taq* DNA polymerase	Promega	USA
6.	dNTP mix	Gene System	USA
7.	$(NH_4)_2SO_4$ (Cat. A4418)	Sigma	USA
8.	Tween 20 (Cat. P9416)	Sigma	USA
9.	1M $MgCl_2$ (Cat.M1028)	Sigma	USA
10.	Tris HCl (Cat. T7149)	Sigma	USA
11.	LE agarose	Seakem	USA
12.	Tris Borate EDTA	Ameresco	USA
13.	Orange G (Cat. O3756)	Sigma	USA
14.	Ficoll (Cat. F2637)	Sigma	USA
15.	EDTA (Cat. E5134)	Sigma	USA
16.	100 bp DNA ladder	Promega	USA
17.	Ethidium Bromide (Cat. E1510)	Sigma	USA

Appendix C

Equipment

1.	High speed centrifuge (Model 5417C)	Eppendorf	Germany
2.	Thermo Block (Model TB02T)	Mit	Thailand
3.	Mixer (Model G560E)	Scientific Industries	USA
4.	UV spectrophotometer (Model UV-1201)	Shimadzu	Japan
5.	Thermal cycler (Model PTC-200)	MJ Research	USA
6.	Electrophoresis (Model Gel Mate 2000)	TOYOBO	Japan
7.	UV transilluminator (Model TVC-312R/F)	Spectroline	USA
8.	Polaroid camera	Gel Cam	UK
9.	Camera hood (Model CH-1013)	Spectroline	USA
10.	Electronic balance (Model L-420P)	Sartorious	Germany
11.	Adjustable automatic pipette (pipetman)	Gilson	USA

Appendix D

Supplies

1.	0.2 mL thin wall 8 tube strip & caps (Cat. 16330)	Sorenson	USA
2.	30 µL filter tip (Cat. 14210)	Sorenson	USA
3.	100 µL filter tip (Cat. GT-99451)	Bioline	USA
4.	10 µL filter tip (Cat.7501-96R-10F)	Evergreen Scientific	USA
5.	3.5 mL sterile transfer pipette (Cat. 222-1590-021)	Evergreen Scientific	USA
6.	1.5 mL microcentrifuge tube (Cat. 17040)	Sorenson	USA
7.	200 µL filter tip (Cat. 7511-96R-250F)	Evergreen Scientific	USA
8.	1000 µL filter tip (Cat. 7515-100R-1000F)	Evergreen Scientific	USA
9.	200 µL tip (Cat. TR-222-C)	Axygen	USA

Appendix E

Manuscript submission

From : <MManigly@usa.redcross.org>
Sent : Wednesday, May 26, 2004 8:20 PM
To : <pawinee_moo@hotmail.com>
Subject : RE: Submitted Manuscript

Thank you for your manuscript submission – you will be hearing from Mary McGinniss shortly.

Marge

Margaret C. Manigly

Office Manager

Technical Services/NRLBGS

Phone: 215-451-4162

FAX: 215-451-2538

-----Original Message-----
From: pawinee kupatawintu [mailto:pawinee_moo@hotmail.com]
Sent: Tuesday, May 25, 2004 8:34 PM
To: Manigly, Margaret
Subject: Submitted Manuscript

May 26, 2004

Mary H. McGinniss,

Managing Editor, *Immunohematology*

10262 Arizona Circle, Bethesda, MD 20817

USA

Dear Ms. Mary H. McGinniss,

We would like to submit our manuscript entitled “Gene frequencies of the HPA-1 to 6 and Gov human platelet antigens in Thai blood donors” to Immunohematology Journal**.**

We have attached manuscript file for your kind consideration.

The original manuscript and 2 copies were sent to you by mail.

We look forward to hearing for your positive response.

Sincerely yours,

Pawinee Kupatawintu

National Blood Centre, Thai Red Cross Society

Henri-Dunant Road, Pathumwan Bangkok 10330

Thailand

Tel & Fax: 662-2556925

E-mail: *pawinee_moo@hotmail.com*

From : <MManigly@usa.redcross.org>
Sent : Friday, June 4, 2004 2:37 AM
To : <pawinee_moo@hotmail.com>
Subject : Pawinee Kupatawintu.doc

Attachment : PawineeKupatawintu.doc (0.05 MB)

Good afternoon,

Attached you will find correspondence from Mary McGinniss regarding your recent submission of your manuscript for Immunohematology.

Sincerely,

Marge

Margaret C. Manigly

Office Manager

Technical Services/NRLBGS

Phone: 215-451-4162

FAX: 215-451-2538

June 3, 2004

Dear Pawinee Kupatawintu,

Your manuscript entitled: Gene frequencies of the HPA-1 to 6 and GOV human platelet antigens in Thai blood donors

Has been received and will be reviewed by members of the editorial staff and by peer reviewers.

After these reviews, you will be notified of our decision concerning your manuscript. This may take up to eight weeks. Please sign and return the enclosed copyright form. Thank you for your support.

Sincerely,
Mary H. McGinniss
Managing Editor
10262 Arizona Circle
Bethesda, MD 20817
Phone & Fax: (301) 299-7443

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Signature of Author

P. Kupatawintu, Bsc, et al

Print Name of Author

Address: Nat. Blood Centre, Thai Red Cross Society
Henri Dunant Road, Pathumwan
Bangkok 10330, Thailand

Please return to: Mary H. McGinniss
Managing Editor, Immunohematology
10262 Arizona Circle
Bethesda, MD 20817
FAX: 301-299-7443

You may fax back just this page after signing.

Or e-mail to mmanigly@usa.redcross.org

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Sent : Thursday, July 29, 2004 1:25 AM
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Subject : Re: your manuscript

| | | my thesis | Inbox

Dear Sir,

Your excellent platelet paper has been successfully peer reviewed and your paper will be published in the March 2005 issue. You will receive before then the final edited paper.

I am sorry for the long delay before publishing, but for 2004 (our 20th anniversary year), 18 invited review papers were (and will be also in December) published in March June and September. I hope this is acceptable to you.

Thank you,

Mary McGinniss

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

P. KUPATAWINTU[1,2], O. NATHALANG[3], R. O-CHAROEN[2], AND

P. PATMASIRIWAT[4]

[1] Department of Clinical Immunology, Faculty of Medical Technology, Mahidol University,

[2] National Blood Centre, Thai Red Cross Society,

[3] Department of Pathology, Phramongkutklao College of Medicine,

[4] Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

Correspondence: Pawinee Kupatawintu

National Blood Centre, Thai Red Cross Society

Henri Dunant Road, Pathumwan

Bangkok 10330, Thailand

Tel & Fax: 662-2556925

E-mail: pawinee_moo@hotmail.com

Running title: HPA-1 to 6 and Gov genotyping in Thais

Abstract

Human platelet alloantigens (HPA) are important in neonatal alloimmune thrombocytopenia (NAIT), post transfusion purpura (PTP), platelet transfusion refractoriness, passive alloimmune thrombocytopenia and transplantation-associated alloimmune thrombocytopenia**.** Thus, HPA genotyping is essential in patient diagnosis and treatment. In this study, we analyzed HPA-1 to 6 and Gov alleles using PCR with sequence specific primers (PCR-SSP) in 500 Thai blood donors, who have been HLA class I antigen-typed**.** It was found that 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% and 44.0%, respectively. In addition, for the Gov system, Gov^a and Gov^b showed frequencies of 49.1 % and 50.9%, 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. Furthermore, this study is also reported on the GFs of Gov^a and Gov^b, which are comparable to those obtained from Asian populations. Our results would 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.

Key words: Human platelet antigen (HPA), PCR-SSP, Thais, Gene frequencies

Introduction

Human platelet alloantigen (HPA) becomes 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 is a common problem in multitransfused patients, which is defined as a low corrected count increment after platelet transfusion. Immunological refractoriness to platelet transfusion is caused by alloantibodies reacting with transfused antigens, such as ABO, HLA class I, and HPA. 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 crossmatching 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% and 6.13% of patients, respectively. In addition, 40% of HPA antibodies could not be identified due to the low availability of HPA-genotyped panels.[8] Having a panel of HLA and HPA-typed donors would be helpful.[4] Different polymerase chain reaction (PCR) techniques have been introduced such as PCR with restriction fragment length polymorphism (PCR-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 aims to analyze HPA-1 to 6 and Gov alleles using PCR-SSP in Thai blood donors, who have been HLA class I antigen-typed and to select HPA-genotyped panels for identification of HPA antibodies in routine testing.

Materials and Methods

Subjects

Five hundred non-repeated 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 sample comprised 369 males and 131 females with ages ranging from 19 to 58 years, the mean age was 36 years. Three milliliters of EDTA blood was collected and informed consent was obtained from all subjects.

Methods

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 the whole blood of the subjects by the salting out technique (Pel-Freez, Brown Deer, WI, USA). The PCR-SSP for HPA-1 to 6 and Gov Systems was performed as previously described,[9,15,16] with some modification. In order to increase specificity for detection of HPA-3b, 2 bases were extended at 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, USA). PCR amplifications were carried out in a GeneAmp PCR System 2700 (Applied

Biosystems, Foster City, CA, USA) or PTC-200 (MJ Research, Waltham, MA, USA). The cycle parameters of the PCR program were one cycle: 96°C for 1 min; five cycles: 96 °C for 25 s, 68 °C for 45 s, 72 °C for 30 s; twenty-eight cycles: 96 °C for 25 s, 61 °C for 45 s, 72 °C for 30 s and one cycle: 72°C for 3 min. The amplified products were electrophoresed through 2.0 percent agarose gel containing 0.5 μg/ml ethidium bromide. The gel was run at 100 V for 30 min 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, Gov^a and Gov^b SSP-typing to test 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 x *af*[a] x *af*[b] x N for heterozygotes and *af*[a or b]^2 x 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 Fisher's exact test.

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 non-repeated Thai blood 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% and 44.0%, respectively. In addition, for the Gov system, Gov^a and Gov^b showed frequencies of 49.1 % and 50.9%, respectively.

In order to test the reproducibility of the assay, 10 DNA samples were randomly selected and tested for HPA-1 to 6 and Gov typing. The results of the repeated assay were similar to the first round of testing. Moreover, this PCR-SSP of HPA typing method was validated using 20 HPA reference materials typed by alternative techniques and in other laboratories. The results showed a 100% concordance.

A comparison of HPA-1 to 6 and Gov gene frequencies among Asian and Caucasian studies is presented in Tables 3 and 4.[6, 12,17-25] The HPA-1 to 6 frequencies in Thai blood donors are more similar to Northeastern Thais (NET) and those of other Asians rather than those of Caucasians. The Gov^a and Gov^b frequencies in our study showed similar results to the previous study[25] but were significantly different from the study in UK populations ($p<0.01$).[23]

Discussion

The investigation of HPA gene and genotype frequencies is important in both population studies and clinical transfusion practice where HPA-typed platelets may be required for alloimmunized patients. Providing more data in 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.[11,21] 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 misinterpretation 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 for either alloimmune thrombocytopenia or in genetic population studies.[4,6,9,10,12,14,19]

Furthermore, the distribution of HPA- 1 to 6 phenotypes in Thai blood donors living in Bangkok are 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 Japanese populations.[23,26-29]

The Gov antibodies have been reported for their clinical importance especially in NAIT, PTP and platelet refractoriness.[23,30] Although the incidence of Gov antibodies was lower than the HPA-1 system, the incidence was equal to HPA-5 system antibodies in Caucasians. The genotype frequencies of the Gov system in this study are comparable to previous studies in Asian populations.[21] 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 in order to provide HPA-matched platelet donors and increase the capability for platelet alloantibody investigation.

Acknowledgements

We would like to thank Dr. Paul Metcalfe, National Institute for Biological Standard 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.

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Author information

1. Pawinee Kupatawintu, B.Sc (Medical Technology)
National Blood Centre, Thai Red Cross Society
Henri Dunant Road, Pathumwan
Bangkok 10330, Thailand
Tel & Fax: 662-2556925
E-mail: pawinee_moo@hotmail.com
2. Oytip Nathalang, Ph.D.
Department of Pathology, Phramongkutklao College of Medicine,
Rajavithi Road, Rajthevi,
Bangkok 10400, Thailand
Tel & Fax: 662-3547791
3. Rachanee O-Charoen, M.D.
National Blood Centre, Thai Red Cross Society
Henri Dunant Road, Pathumwan
Bangkok 10330, Thailand
Tel: 662-2524106
Fax: 662-2555558
4. Pimpicha Patmasiriwat, Ph.D.
Department of Clinical Microscopy, Faculty of Medical Technology,
Mahidol University, Bangkoknoi,
Bangkok 10700, Thailand
Tel: 662-4197169

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
HGH control	5′ TCA CGG ATT TCT GTT GTG TTT C 3′	429	0.4 μM
			HPA-5 0.1 μM
			Gov 0.2 μM

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

Genotype	No.	Genotype frequencies (%)	Gene frequencies	
			Gene	(%)
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		

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

HPA	Thai (N=500)	NET[6] (N=300)	Taiwan[19] (N=300)	Hong Kong[17] (N=100)	Korea[18] (N=100)	Japan[20] (N=331)	Spain[24] (N=107)	Wales[12] (N=392)	Australia[22] (N=1,000)	Norway[25] (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: Thai blood donors, NET: Northeastern Thais, NA: 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[a]	Norway[25]	UK[23]	Taiwan[21]	Indonesia[21]	Thai[21]	Filipino[21]
	(N=500)	(N=105)	(N=113)	(N=566)	(N=107)	(N=137)	(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

[a]Thai: Thai blood donors; [b]P< 0.01

Fig. 1 A representative case for 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, USA).

BIOGRAPHY

NAME	Miss.Pawinee Kupatawintu
DATE OF BIRTH	31 August 1967
PLACE OF BIRTH	Khonkaen, Thailand
INSTITUTIONS ATTENDED	Chulalongkorn University, 1989: Bachelor of Science (Medical Technology)
POSITION&OFFICE	National Blood Centre, Thai Red Cross Society Bangkok, Thailand Position: Head of department Tel. 662-2556925 E-mail: pawinee_moo@yahoo.com
HOME ADDRESS	25/7 M8 Soi Samakompath Nawamin Road, Bungkum Bangkok 10230 Thailand Tel. 662-9467578