Comparison of a Simple-Probe Real-Time PCR and Multiplex PCR Techniques for HPA-1 to HPA-6 and HPA-15 Genotyping

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Background: We aimed to compare HPA-1 to HPA-6 and HPA-15 genotyping results obtained by a simple-probe real-time polymerase chain reaction (PCR) technique with the multiplex PCR technique. **Methods:** Five hundred DNA samples from the Thai National Stem Cell Donor Registry (TSCDR) of the National Blood Centre, Thai Red Cross Society were included. Human platelet antigen (HPA) genotyping was performed by simple-probe real-time PCR and multiplex PCR techniques. **Results:** HPA-1, HPA-2, HPA-3, and HPA-4 genotyping results obtained by both techniques were in agreement. The misinterpretation of HPA-5, HPA-6, and HPA-15 genotypes was found in eight samples by simple-probe real-time PCR and HPA genotypes were confirmed by DNA sequencing. Two samples of HPA-5 were misinterpreted as HPA-5a5a instead of HPA-5a5b

due to an NM_002203.3:c.1594A>C mutation (rs199808499) near the HPA-5 polymorphism (5' side). Five samples of HPA-6a6b were misinterpreted as HPA-6b6b because of an NM_000212.2:c.1545G>A mutation (rs4634) adjacent to the HPA-6 polymorphism (3' side). Interestingly, one sample of HPA-15a15b was misinterpreted as HPA-15b15b due to an NM_133493.1:c.2118C>A mutation near the HPA-15 polymorphism (3' side). **Conclusions:** HPA genotyping results by two PCR techniques were compared. Incorrect assignments were found due to genetic variations near each HPA single nucleotide polymorphism. Therefore, to avoid false assignment, the use of two genotyping techniques is recommended. J. Clin. Lab. Anal. 29:94–99, 2015. © 2014 Wiley Periodicals, Inc.

Key words: HPA; genotyping; multiplex PCR; simple-probe real-time PCR; Thais

INTRODUCTION

At present, human platelet antigen (HPA) genotyping is commonly used to confirm the detection of HPA alloantibodies in patients with platelet transfusion refractoriness, posttransfusion purpura, and fetal and neonatal alloimmune thrombocytopenia (1). Additionally, HPA genotyping in large number of blood donors is necessary to provide HPA-matched platelet for those patients. The Platelet Working Party of the International Society of Blood Transfusion (ISBT) classifies the HPA systems numerically and alphabetically to reflect the system (2), and currently 27 HPA systems are reported (for further information see <http://www.ebi.ac.uk/ipd/hpa/>). However, only HPA-1 to HPA-6 and HPA-15 genotyping is rou-

tinely performed in most laboratories due to their clinical significance.

In general, most HPA allele polymorphisms can be distinguished due to a single nucleotide polymorphism (SNP). Several techniques have been established for HPA genotyping, such as polymerase chain reaction (PCR) with sequence-specific primer (PCR-SSP) (3, 4), PCR

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with restriction fragment length polymorphism (PCR-RFLP) (5), real-time PCR (6–12), and DNA sequencing based typing (13–15). Although the PCR-SSP is widely used for HPA genotyping because of its simplicity and cost effectiveness, a previous study demonstrated that multiplex PCR could provide greater convenience at half the cost of PCR-SSP (16). The disadvantages of PCR-SSP and multiplex PCR include being labor intensive and being unsuitable for high-throughput HPA genotyping (13).

To provide high-throughput HPA genotyping, real-time PCR techniques such as real-time PCR with hybridization probe (6, 7), real-time PCR with TaqMan probe (8–10), real-time PCR with high-resolution amplicon melting analysis (11), and real-time PCR with displacing probes (12) have been implemented. Nevertheless, the real-time PCR technique has a limitation for HPA-6 genotyping because of a 1545 G>A polymorphism (rs4634) adjacent to the 1544G>A SNP, as previously described (11). Moreover, an additional limitation for HPA-5 genotyping by BeadChip Technology (BioArray, Immucor, Paris, France) due to a 1594A>C SNP (rs199808499) near the 1600 G>A SNP was recently reported (17). The purposes of this study are to compare the results of HPA-1 to HPA-6 and HPA-15 genotyping obtained by a simple-probe real-time PCR technique with the multiplex PCR technique and to determine whether a simple-probe real-time PCR can be used to screen platelet donors.

MATERIALS AND METHODS

Study Population

Five hundred blood group O DNA samples consisted of 300 samples from our previous study (16) and an additional 200 samples were from the Thai National Stem Cell Donor Registry (TSCDR) of the National Blood Centre, Thai Red Cross Society. Informed consent was obtained from each subject. This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. All DNA samples were stored at -20°C until use.

DNA Standards

Known HPA-1 to HPA-6 and HPA-15 DNA samples were provided by the Australasian Platelet Antibody Workshop (2001–2010).

Primers

Primers for multiplex PCR were identical to those previously described (16). All primers and hybridization probes for a simple-probe real-time PCR technique were commercially provided by Tib Molbiol (Berlin, Germany).

METHODS

HPA Genotyping by Multiplex PCR Technique (16)

Briefly, PCR reaction mixtures of six multiplex sets for *HPA-1* to *HPA-6* and *HPA-15* genotyping consisted of 5 μl of $2\times$ PCR master mix (ABgene, Surrey, UK), 0.5 μl of PCR-grade water, 2.5 μl of primer mix, and 2 μl (50–150 ng) of genomic DNA. PCR amplification of six multiplex sets was performed in a Thermo Cycler PTC-200 (MJ Research, Waltham, MA). The PCR program for all multiplex sets consisted of one cycle of 96°C for 1 min; five cycles of 96°C for 25 sec, 66°C for 45 sec, 72°C for 30 sec; 25 cycles of 96°C for 25 sec, 60.5°C for 45 sec, 72°C for 30 sec; and one cycle of 72°C for 3 min. The PCR products were separated in 2% agarose gel in $0.5\times$ tris borate ethylenediamine tetraacetic acid containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide for 12 min at 100 V and visualized under an ultraviolet (UV) transilluminator for HPA allele assessment.

HPA Genotyping by a Simple-Probe Real-Time PCR Technique

PCR of *HPA-1* to *HPA-6* and *HPA-15* genotyping was performed in a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). Generally, 10 μl reaction mixtures contained 5.7 μl of PCR-grade water, 0.5 μl of Reagent Mix containing all primers and probes, 1 μl of $10\times$ LightCycler FastStart DNA Master HybProbe, 0.8 μl of MgCl_2 (3.0 mM), and 2 μl of genomic DNA (50–100 ng). The PCR program for *HPA-1* to *HPA-6* and *HPA-15* genotyping consisted of one cycle of denaturation: 95°C for 10 min; 45 cycles of quantification: 95°C for 10 sec, 60°C for 10 sec, 72°C for 15 sec; one cycle of melting curves: 95°C for 30 sec, 40°C for 2 min and gradually increased to 75°C with continuous acquisition mode. The fluorescent signal is proportional to the amount of specific PCR product generated. After completing amplification, *HPA-1* to *HPA-6* and *HPA-15* genotyping results were interpreted using melting curve analysis by LightCycler 480 software version 1.5.0.39 (Roche Diagnostics). HPA genotyping for the simple-probe real-time PCR technique was based on the melting temperature (T_m) calculated from the derivative melting temperature plots of the LightCycler data. An a/a homozygous allele has a single peak at a certain T_m , while a b/b homozygous allele has a single peak at a different T_m , and an a/b heterozygous allele has both peaks. The T_m characteristics of *HPA-1* to *HPA-6* and *HPA-15* alleles are shown in Table 1.

In addition, DNA sequencing was performed according to previous studies (14, 15) to clarify the discrepant results of HPA genotyping obtained by multiplex PCR and simple-probe real-time PCR techniques.

TABLE 1. Melting Temperature (T_m) of HPA-1 to HPA-6 and HPA-15 Alleles Obtained From Simple-Probe Real-Time PCR

HPA	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b	15a	15b
T_m (°C)	52.7	57.5	65.2	54.7	63.8	55.5	65.1	58.0	58.0	49.9	61.9	67.8	62.8	54.8

RESULTS

Validity of *HPA-1* to *HPA-6* and *HPA-15* genotyping by multiplex PCR and simple-probe real-time PCR techniques were performed using known *HPA-1* to *HPA-6* and *HPA-15* DNA samples. *HPA-1* to *HPA-6* and *HPA-15* genotyping results of 500 DNA samples obtained by a simple-probe real-time PCR and multiplex PCR techniques were compared. *HPA-1*, *HPA-2*, *HPA-3*, and

HPA-4 genotyping results obtained by both techniques were in 100% concordance. However, the discrepant results of *HPA-5*, *HPA-6*, and *HPA-15* genotypes were found in eight samples: two samples were typed as *HPA-5a5a*, five samples were typed as *HPA-6b6b*, and one sample was typed as *HPA-15b15b* by simple-probe real-time PCR, whereas they were typed as *HPA-5a5b*, *HPA-6a6b*, and *HPA-15a15b*, respectively, by multiplex PCR (Fig. 1,

Fig. 1. Melting curve analysis of HPA-5, HPA-6, and HPA-15 from simple-probe real-time PCR technique.

Tables 2 and 3). DNA sequencing was performed on these samples. For two samples with *HPA-5* genotyping discrepancy, a 1600G>A was confirmed as heterozygous *HPA-5a5b* and revealed an NM_002203.3:c.1594A>C mutation (rs199808499) near the *HPA-5* polymorphism (5' side) as shown in Figure 2. Regarding *HPA-6* genotyping discrepancy in five samples, a 1544G>A was confirmed as heterozygous *HPA-6a6b* and revealed an NM_000212.2:c.1545G>A mutation (rs4634) adjacent to the *HPA-6* polymorphism (3' side) as shown in Figure 2. Interestingly, in one sample with a *HPA-15* genotyping discrepancy, a 2108C>A was confirmed as heterozygous *HPA-15a15b* and revealed an NM_133493.1:c.2118C>A mutation near the *HPA-15* polymorphism (3' side), as shown in Figure 2.

In addition, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the simple-probe real-time PCR technique were compared with the multiplex PCR technique for the detection of *HPA-1* to *HPA-6* and *HPA-15* alleles. It was found that the sensitivity and specificity of the simple-probe real-time PCR were 99.9% and 99.2%, respectively. The positive predictive value, negative predictive value, and accuracy of the simple-probe real-time PCR were 99.9%, 99.2%, and 99.8%, respectively.

TABLE 2. Comparison of HPA-1 to HPA-6 and HPA-15 Genotyping Results Obtained by Real-Time PCR and Multiplex PCR Techniques (N = 500)

HPA genotype	Real-time PCR	Multiplex PCR
<i>HPA-1a1a</i>	479	479
<i>HPA-1a1b</i>	20	20
<i>HPA-1b1b</i>	1	1
<i>HPA-2a2a</i>	458	458
<i>HPA-2a2b</i>	40	40
<i>HPA-2b2b</i>	2	2
<i>HPA-3a3a</i>	171	171
<i>HPA-3a3b</i>	230	230
<i>HPA-3b3b</i>	99	99
<i>HPA-4a4a</i>	499	499
<i>HPA-4a4b</i>	1	1
<i>HPA-4b4b</i>	0	0
<i>HPA-5a5a</i>	473 ^a	471
<i>HPA-5a5b</i>	27 ^a	29
<i>HPA-5b5b</i>	0	0
<i>HPA-6a6a</i>	484	484
<i>HPA-6a6b</i>	11 ^b	16
<i>HPA-6b6b</i>	5 ^b	0
<i>HPA-15a15a</i>	134	134
<i>HPA-15a15b</i>	272 ^c	273
<i>HPA-15b15b</i>	94 ^c	93

^aTwo samples were confirmed as *HPA-5a5b*.

^bFive samples were confirmed as *HPA-6a6b*.

^cOne sample was confirmed as *HPA-15a15b*.

Fig. 2. Chromatogram of mutation sequencing analysis, (A) and (B) *HPA-5a5b*; (C) and (D) *HPA-6a6b*; (E) and (F) *HPA-15a15b*.

TABLE 3. Mutations of HPA-5, HPA-6, and HPA-15 Discrepant Results

HPA	Genotyping results by		HPA SNP and adjacent SNP		Amino acid displacement	
	Multiplex	Real time				
HPA-5			DNA sequence in <i>ITGA2</i> gene		Amino acid position in GPIa	
			1594	1600 (HPA-5)	503	505
Reference HPA-5a5b	<i>HPA-5a5b</i>	<i>HPA-5a5b</i>	A	G/A	Ile	Glu/Lys
No. 374 and 578	<i>HPA-5a5b</i>	<i>HPA-5a5a</i>	A/C	G/A	Ile/Leu	Glu/Lys
HPA-6			DNA sequence in <i>ITGB3</i> gene		Amino acid displacement	
			1544 (HPA-6)	1545	489	
Reference HPA-6a6b	<i>HPA-6a6b</i>	<i>HPA-6a6b</i>	G/A	G		Arg/Gln
No. 224, 296, 386, 429, and 442	<i>HPA-6a6b</i>	<i>HPA-6b6b</i>	G/A	G/A		Arg/Gln
HPA-15			DNA sequence in <i>CD109</i> gene		Amino acid position in CD109	
			2108 (HPA-15)	2118	703	706
Reference HPA-15a15b	<i>HPA-15a15b</i>	<i>HPA-15a15b</i>	C/A	C	Ser/Tyr	Tyr
No. 273	<i>HPA-15a15b</i>	<i>HPA-15b15b</i>	C/A	C/A	Ser/Tyr	Tyr/stop codon

DISCUSSION

Currently, various HPA genotyping techniques have been used for routine testing in most laboratories. According to the report on the 15th International Society of Blood Transfusion Platelet Immunology Workshop, 31 laboratories were participating in HPA genotyping using different techniques such as PCR-SSP, PCR-RFLP, TaqMan, sequencing, and others. Six DNA samples were provided to be tested for *HPA-1*, *HPA-2*, *HPA-3*, *HPA-4*, *HPA-5*, *HPA-6*, *HPA-9*, and *HPA-15* genotyping. It was found that most laboratories (77%) were able to correctly type all six DNA samples for HPA genotyping. Moreover, typing errors were found in four of 31 laboratories in a DNA sample with a rare mutation within the binding region of the primer (18). Therefore, selection of appropriate techniques for HPA genotyping should be considered.

At present, multiplex PCR is implemented for HPA genotyping at the National Blood Centre, Thai Red Cross Society. Although this technique is simple and cost effective, it is unsuitable for screening the large numbers of donors needed to provide platelet panel selection and establish a HPA-typed donor database. The utilization of a simple-probe real-time PCR technique is helpful to reduce labor-intensive and could provide better throughput HPA genotyping than multiplex PCR. However, for comparing the HPA genotyping results, routinely used techniques are recommended. The results of this study confirm the need for cross-checking with multiple techniques.

In this study, comparison of HPA genotyping results between multiplex PCR and simple-probe real-time PCR techniques in 500 DNA samples showed that *HPA-1*, *HPA-2*, *HPA-3*, and *HPA-4* genotyping results were in agreement. Interestingly, eight discordant results were found in *HPA-5*, *HPA-6*, and *HPA-15* genotyping. Therefore, DNA sequencing was performed in all samples. Regarding HPA-5 discrepant results ($n = 2$), *GPIa* exon 13 se-

quencing confirmed the *HPA-5a5b* genotype and revealed 1594A>C polymorphism (rs199808499) in both samples. This mutation near the HPA-5 polymorphism (5' side) could lead to an Ile503Leu amino acid change as previously described (17). Additionally, in five samples of HPA-6 discrepant results, a 1545G>A polymorphism (rs4634) adjacent to the 1544G>A SNP was demonstrated in all samples. Concerning *HPA-6b6b* genotype, a rare genotype among Thai populations (3, 19), even though this silent mutation does not affect the amino acid change, it can cause an incorrect interpretation from *HPA-6a6b* to *HPA-6b6b* using the simple-probe real-time PCR technique. Similar findings were obtained by melting curve analysis of *HPA-6* genotype using HybProbe assay (11).

Importantly, an extraordinary melting curve analysis result was found in a sample of *HPA-15a15b* with 2118C>A (Fig. 1). The genotyping result was *HPA-15b15b* by simple-probe real-time PCR, while it was *HPA-15a15b* by multiplex PCR. The NM_133493.1:c.2118C>A nonsense mutation could change amino acid from tyrosine to stop codon. Due to the unavailability of standardized anti-HPA-15 reagents to perform HPA phenotyping, it was difficult to investigate antigen expression and its function in platelet alloimmunization as well as the clinical effect of this mutation. Since the *HPA-15a15b* is the most common genotype in Thai (48.2%) and Chinese populations (50.2%), followed by *HPA-15a15a* (26.8% and 28.1%) and *HPA-15b15b* (25.0% and 21.7%), respectively (3, 4), only the gene frequency cannot realize misinterpretation. From this study, it appears that the simple-probe real-time PCR technique offers an overall sensitivity of 99.9% and a specificity of 99.2% for the detection of the *HPA-1* to *HPA-6* and *HPA-15* alleles. However, the interpretation of HPA genotyping following automatic software analysis, manually checked by laboratory personnel is suggested in order to identify abnormal patterns of melting curve

analysis. Thereafter, reexamined using another reference method is recommended.

Factors involved in selecting HPA genotyping techniques include throughput, cost, time consumption, equipment, and personnel. In addition, consideration of primer and probe design using in-house and commercial kits is needed, especially in genotype discrepancy results of *HPA-1*, *HPA-5*, *HPA-6*, and *HPA-15* (11, 17, 20–22). Therefore, two genotyping techniques using different primer sets should be employed routinely by laboratories to avoid misinterpretation.

In conclusion, HPA genotyping results by multiplex PCR and simple-probe real-time PCR techniques were compared. Misinterpretations were found in *HPA-5*, *HPA-6*, and *HPA-15* genotypes, caused by genetic variations near each HPA SNP. To avoid false typing assignment, the alternative use of two genotyping techniques is recommended.

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CONFLICT OF INTEREST

The authors have no disclaimers to make or conflicts to disclose.

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