Report

Genotyping human platelet alloantigens (HPA 1–5) in Saudis from Eastern Province, Saudi Arabia

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SUMMARY In this study we report for the first time the distribution of human platelet alloantigens (HPA) in Saudis. These antigens are implicated in the pathophysiology of alloimmune thrombocytopenia. We collected blood samples from 84 healthy male Saudi blood donors. DNA isolated by salting-out and ethanol precipitation was amplified for genes HPA 1–5 using the polymerase chain reaction/sequence specific primer method. We found high HPA-1 polymorphism similar to Caucasians. HPA-4 polymorphism in Saudis was, however, greater than in Caucasians, and more similar to that of the Japanese. These results suggest that both these two HPA systems may be clinically important in Saudis.

Introduction

More than 50 platelet membrane glycoproteins (gp) have been identified [*1*]. These gp are important for platelet function. Traditionally they have been classified according to their electrophoretic mobility, function and reactivity with respective monoclonal antibodies. Recently these have been recognized as members of the integrin family, and are classified according to their β and α units [*2*] (Table 1). Deletions, insertions, non-sense substitutions and, less commonly, mis-sense substitutions of platelet gp genes result in abnormal platelet function and bleeding [*3*]. Among the different platelet gp, a few are polymorphic (Table 1). This polymorphism is not associated with any alteration in platelet function [*1*], but can initiate immune response and result in alloimmune thrombocytopenia (AIT), such as neonatal alloimmune thrombocytopenia (NAIT), post-transfusion purpura and refractoriness to platelet transfusions.

Platelet gp polymorphism was recognized in the late 1950s [*4*]. In 1979, Pl^A alloantigen was localized on platelet gp IIIa [*5*]. Our knowledge has rapidly expanded in the past 10 years. The complete protein sequence of gp IIIa was deduced from c-DNA in 1987 [*6*]. The following year, the gene was localized to chromosome 17 [*7*]. *Taq* I restriction enzyme polymorphism was recognized in the gp IIIa gene in 1988 [*8*]. In the following few years, the protein structure and DNA sequence underlying polymorphisms in human platelet alloantigens (HPA), HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, HPA-6, HPA-7 and HPA-8, were described [*9–16*]. It was found that in all of these systems, the polymorphism is under-

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Table 1 **Classifications of platelet membrane glycoproteins**

Function	Electrophoretic classification	CD classification	Integrin classification
Collagen receptor	gp 1 a* /119	CD 49 b	$\alpha_2 \beta_1$
Fibronectin receptor	gp 1 c /119	CD 49 e	$\alpha_5 \beta_1$
Vitronectin receptor	–	–	$\alpha_v \beta_3$
Fibrinogen receptor	gp 11 b* /IIIa*	CD 41	$\alpha_{11b} \beta_3$
Laminin receptor	gp 1 c' /11a	CD 49 f	$\alpha_6 \beta_1$
Von Willebrand receptor	gp 1 b* /1X	CD 42 a,b,c	Not an integrin
? Collagen or thrombospondin receptor	gp 1V	CD 36	Not an integrin

**Polymorphic glycoproteins*

lined by a single amino acid substitution as a result of a single base pair substitution. This molecular information allowed the design of novel diagnostic approaches and reclassification of platelet alloantigen systems, as described below.

Traditionally platelet alloantigens were named after the patients in whom the respective antibodies were first isolated. This system of classification was not only confusing, but also led to the use of more than one name for a single alloantigen. An important milestone was the introduction of a system of HPA classification by Von dem Borne and Decary in 1990 [*17*], who classified platelet alloantigens into 9 systems: HPA-1 through to HPA-9 (Table 2). Each of these is biallelic. High and low frequency alloantigens were allocated the letters "a" and "b" respectively. Because of its simplicity, this classification gained wide acceptance and usage. However, because of incomplete knowledge about protein sequence and DNA structure, the system was strongly criticized by Newman, who thought it scientifically inaccurate [*18*].

Newman proposed another classification, based on molecular knowledge. For example, the difference between HPA-1a and HPA-1b is a single amino acid substitution at position 33 (Leu→Pro). Thus, HPA-1b becomes Pro33 gp IIIa. He also suggested modification of the existing HPA classification. Despite this criticism, HPA classification has retained wide usage because of its simplicity [*19*].

Traditionally, typing of HPAs relied on serological methods such as platelet immunofluorescence testing (PIFT), monoclonal antibody specific immobilization of platelet antigens, mixed passive haemagglutination, modified Ag-capture enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation (RIP) and Western blot. These techniques, however, suffer from two limitations; the limited source of typing antisera and the low yield of platelets in throm- bocytopenic patients. Typing HPA by molecular techniques overcomes the limitations of the serological methods just mentioned. Several genotypic techniques were used previously, including polymerase chain reaction (PCR) with allele-specific restriction enzyme analysis (ASRA) [*9,20*] and oligonucleotide typing (ONT) [*21*]. More recently, PCR with se-

Table 2 **Human platelet alloantigen systems**

Von dem Borne 1990	Newman 1994	Serological designation	Electrophoretic classification	Function
HPA-1a	HPA-1a -	Pl[A1]	Gp IIIa	Fibrinogen
-1b	-1b	Pl[A2] (ZW)		receptor
HPA-2a	HPA-2a	Ko[b]	Gp Ib	Von Willebrand
-2b	-2b	Ko[a] (Sib)		receptor
HPA-3a	HPA-3a	Bak[a]	Gp IIb	Fibrinogen
-3b	-3b	Bak[b] (Lek)		receptor
HPA-4a	HPA-1a	Pen[a]	Gp IIIa	Fibrinogen
-4b	-1c	Pen[b] (Yuk)		receptor
HPA-5a	HPA-5a	Br[b]	Gp Ia	Collagen
-5b	-5b	Br[a] (Hc)(Zav)		receptor
HPA-6a	HPA-1a	Ca[b](Tu[b])	Gp IIIa	Fibrinogen
-6b	-1e	Ca[a]		receptor
HPA-7a	HPA-1a	Mo[b]	Gp IIIa	Fibrinogen
-7b	HPA-1b	Mo[a]		receptor
HPA-8a	HPA-1a	Sr[b]	Gp IIIa	Fibrinogen
-8b	-1f	Sr[a]		receptor
HPA-9a		Max	Gp IIb	Fibrinogen receptor

quence specific primers [22–25] and multiplex PCR [26] have been introduced.

In our study we chose the PCR-sequence specific primer (SSP) technique, which has the advantage of being a single-step method where the specificity of the primer simplifies downstream detection of the amplified product. Despite the fact that the future introduction of monoclonal antibodies may revolutionize HPA serotyping [27], it will still require sufficient platelet yield. In our opinion, genotyping will be the method of choice in the future.

In this study we evaluated the distribution of HPAs in healthy Saudis. This information allows interpopulation comparisons and could be of anthropological significance. It will also help to anticipate the size and cause of AIT in our community. To our knowledge it is the first study of its type in Saudi Arabia, and probably the first in the Arabic-speaking countries.

Subjects and methods

Blood samples (10 mL of citrate-phosphate-dextrose-adenine (CPDA blood) were collected from 84 healthy, male, Saudi donors attending the Regional Laboratory and Blood Bank in Dammam. Lymphocytes were separated by Ficoll- Hypaque. DNA extraction was performed by salting out and ethanol precipitation. DNA

amplification was by the PCR-SSP method, as recommended by Metcalfe et al. and Cavanagh et al. [22,25]. For each HPA system, a set of one common primer and two allele-specific primers were used (Table 3). In addition, a pair of positive control primers for the human growth hormone (HGH) gene were also included as quality control for the different stages of DNA extraction, amplification and detection.

The volume of the PCR reaction mixture was 10 µL It was composed of 2 µL genomic DNA, 0.2 µL deoxyribonucleoside triphosphate (dNTP) mix, 0.09 µL *Taq* polymerase (0.45 U), 1 µL common primer, 1 µL specific primer, 2 µL positive control primer, 1 µL PCR buffer and 2.73 µL water. DNA was amplified in a GeneAmp® PCR System 9600 (PE Biosystems, Foster City, California, United States of America). The amplification included 33 cycles with gradually decreasing annealing temperatures; 68 °C to 61 °C, then 51 °C. Amplified products were detected by ultraviolet illumination after agarose gel electrophoresis. Expected amplification product sizes are shown in Table 3. We varied slightly from Metcalfe's method in the following: a longer denaturation period was included (95 °C for 3 minutes) before the start of amplification; a higher concentration of *Taq* polymerase (0.45 U rather than 0.33 U) was added in the reaction mix; and more amplification product (7 µL compared to 2.7 µL)

Table 3 **Primer sequences used for HPA 1–5 PCR-SSP analysis**

HPA	Specificity	Sequence	Size (mer)	Product size (bp)
HPA-1	1a	5'TCAGGTCACAGCGAGGTGAGGCCA	24	90
	1b	5'TCAGGTCACAGCGAGGTGAGGCCG	24	
	Common	5'CTGCAGGAGGTAGAGAGTCGCCATAG	26	
HPA-2	2a	5'GCCCCCAGGGCTCCTGAC3'	18	258
	2b	5'GCCCCCAGGGCTCCTGAT3'	18	
	Common	5'TCAGCATTGTCCTGCAGCCA3'	20	
HPA-3	3a	5'TGGACTGGGGCTGCCCAT3'	19	267
	3b	5'TGGACTGGGGCTGCCCAG3'	19	
	Common	5'TCCATGTTCACTTGAAGTGCT3'	21	
HPA-4	4a	5'GCTGGCCACCCAGATGCG3'	18	120
	4b	5'GCTGGCCACCCAGATGCA3'	18	
	Common	5'CAGGGGTTTTCGAGGGCCT3'	19	
HPA-5	5a	5'AGTCTACCTGTTTACTATCAAAG3'	23	246
	5b	5'AGTCTACCTGTTTACTCAAAA3'	23	
	Common	5'CTCTCATGGAAAATGGCAGTG3'	21	
Positive	HGH	5'TGGACTGGGGCTGCCCAT3'	21	429
Controls	HGH	5'TGGACTGGGGCTGCCCAG3'	22	

PCR-SSP = polymerase chain reaction/sequence specific primer

was added to the electrophoretic well. Another important modifi- cation was the use of longer primers (24-mers) and higher annealing temperatures for HPA-1 amplification (see below). Results were considered reliable only when positive control primers showed amplification and the PCR product was of the expected size.

Results

The most common HPA genotypes in Saudis were HPA-1(a+b-), HPA-2(a+b-), HPA-3(a+b-), HPA-4(a+b-), HPA-5(a+b-) (Table 4). Figure 1 shows an example of gel electrophoresis results with HPA genotype similar to that described above. Amplified products were of the expected size. Fast bands were attributed to excess primers remaining after amplification. The least robust amplification was obtained with HPA-5 typing followed by HPA-3. These two systems required optimal conditions for amplification. In contrast, HPA-1 amplified with ease. In fact, we think there was over-amplification in tube 1b. Our initial typing suggested a high frequency of the HPA-1b gene because of the high percentage of HPA-1 heterozygotes (81%). However, the absence of homozygotes for HPA-1b (0%) indicates the opposite. We propose two explanations for this contradiction. The first could be a technical error, with mislabelling or non-deliberate contamination of the "b" tube with "a" primer. However, this possibility was excluded by repeat testing and by the preparation of a new, master PCR mix. A second possible explanation is the occurrence of non-specific amplification of HPA-1a by 1b primer. We therefore tried to improve PCR-SSP specificity. We reviewed the concentration of magnesium, various primers and *Taq* polymerase in the final reaction mix. All

Table 4 **Distribution of HPA specificities in Saudi, Dutch and Japanese populations**

HPA	Caucasians[1] (%)	Japanese[2] (%)	Saudis[3] (%)
HPA-1			
(a+,b-)	65.0	98.0	60.0
(a+,b+)	32.0	2.0	40.0
(a-,b+)	3.0	< 1.8	0.0
HPA-2			
(a+,b-)	83.0	82.0	65.0
(a+,b+)	17.0	16.0	31.0
(a-,b+)	< 0.5	1.8	4.0
HPA-3			
(a+,b-)	24.0	56.0	81.0
(a+,b+)	56.0	31.0	14.0
(a-,b+)	20.0	13.0	5.0
HPA-4			
(a+,b-)	100.0	100.0	96.0
(a+,b+)	< 0.5	< 1.8	2.0
(a-,b+)	< 0.5	< 1.8	2.0
HPA-5			
(a+,b-)	83.0	95.0	72.0
(a+,b+)	15.0	6.0	25.0
(a-,b+)	< 0.5	< 1.8	3.0

[1]*Multiplex PCR, 220 samples [26]*
[2]*Multiplex PCR, 55 samples [26]*
[3]*PCR-SSP, 84 samples (present study)*

were found to be within optimal range. Deliberately introduced destabilizing mismatches in primer design are known to enhance PCR specificity. These were already included in our HPA-1 primers. In addition, a higher annealing temperature and longer primers are also known to improve PCR specificity [*28*]. We tested this hypothesis by comparing the performance of short and long primers with high and low annealing temperatures. When long primers (24-mer rather than 19-mer) were used together with a high annealing temperature (68 °C, compared with 51 °C, 61 °C and 68 °C),

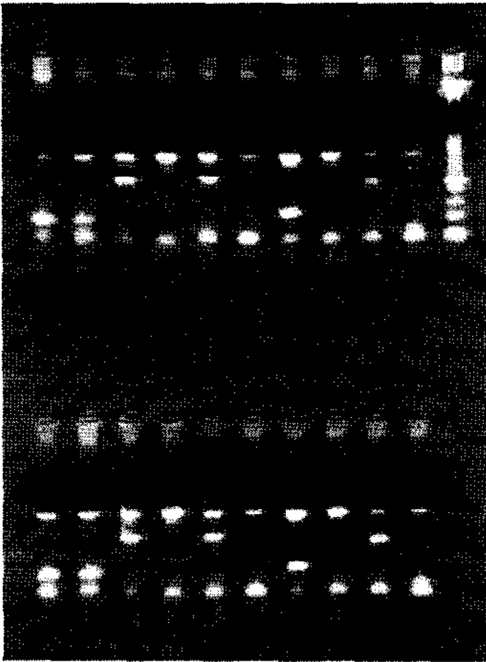

Figure 1 Agarose-gel electrophoresis seen under ultraviolet illuminatin. Well II contains 50 bp ladder. Wells 1 and 2; 3 and 4; 5 and 6; 7 and 8; 9 and 10 are for HPA systems 1, 2, 3, 4 and 5 respectively. Both specimens show similar genotype HPA-1(a+b+), HPA-2(a+b-), HPA-3(a+b-), HPA-4 (a+b-) and HPA-5 (a+b-). Note the slow band due to amplification product of positive control primers (429 6P). Fast bands probably represent excess primers.

nonspecific amplification disappeared. We genotyped the HPA-1 system in 35 Saudi healthy blood donors with the modified technique discussed previously. Homozygosity for HPA-1a accounted for 60%, and heterozygosity for HPA-1a+1b+ was 40%.

Discussion

In our study we used the PCR-SSP method as described by Cavanagh et al. [*25*]. PCR-SSP has the advantage of allowing simultaneous amplification of HPA-1 to HPA-5 using the same PCR set-up. However, we experienced difficulties in HPA-1 genotyping, because of nonspecific amplification. These difficulties were resolved by minor modification of the originally described technique.

Our results for HPA typing generally agree with previously reported studies. Table 4 shows the comparison of HPA distributions between Saudis in our study and results reported by Legler [*26*] using multiplex PCR in Caucasians and Japanese. We found high polymorphism of HPA-1 as well as HPA-4 distribution in Saudis. Saudis, therefore, appear to bear a closer resemblance to Caucasians who are known to have more HPA-1 polymorphisms. In Caucasians, the HPA-1 system is the most important alloantigen system clinically, and it is implicated in AIT. However, HPA-4 polymorphism is also high in Saudis which resembles the Japanese in whom HPA-4 system is clinically more important.

HPA-2 and HPA-5 polymorphisms were also higher in Saudis compared with Caucasians, while HPA-3 was less polymorphic. Theoretically speaking, the higher the degree of polymorphism in an antigen system, the more likely it will be clinically important, although this is not always true; other important contributing factors include immunogenicity and accessibility of the antigen to the antibody once formed. We recently reported a case of NAIT in a Saudi neonate caused by anti-HPA 2b [*29*].

In conclusion, Saudis have a distinct distribution of the HPA system which differs from those of Japanese and Caucasian populations. Except for HPA-3, which

shows less polymorphism in Saudis, all other HPA systems including HPA-1, 2, 4, and 5 are more polymorphic compared with Caucasians.

We intend to extend this study to involve larger numbers of Saudis and to include patients with AIT. This will allow us to evaluate the clinical significance of the different HPA systems in the region.

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