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Genotyping of Human Platelet Antigen-1 to -5 and -15 by Polymerase Chain Reaction with Sequence-specific Primers (PCR-SSP) and Real-time PCR in Azeri Blood Donors

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ABSTRACT

Human platelet antigens (HPAs) are glycoproteins on the platelet surface that a single nucleotide mutation in the coding region gene could lead to the variation of different HPA polymorphisms. These antigens have shown variation among different races and may trigger immune responses during blood transfusion and pregnancy. Genotyping of HPAs is useful for managing these reactions and establishing a platelet registry to decrease platelet transfusion reactions. This study aimed to compare allelic and genotype frequencies of human platelet antigens in the Azeri ethnicity by TaqMan Real-time and polymerase chain reaction with sequence-specific primers (PCR-SSP) methods.

DNA was extracted from the whole blood of 100 Azeri blood donors in the Ardabil Blood Transfusion Center. Genotyping of HPA-1 to -5 and -15 was performed by TaqMan Real-time PCR, and PCR-SSP and consistency of results were evaluated.

The results of PCR-SSP and TaqMan Real-time PCR showed complete consistency. The allele frequencies were 91.5% and 8.5% for HPA-1a and -1b; 88% and 12% for HPA-2a and -2b; 58% and 42 % for HPA-3a and -3b; 100% for HPA-4a; 91% and 9% for HPA-5a and -5b; 56.5% and 43.5% for HPA-15a and -15b alleles.

Not incompatibility was detected in HPAs genotyping by PCR-SSP and TaqMan Real-time PCR so that real-time PCR can be used as a robust and quick method for HPA genotyping. We found differences between Azeri blood donors and previously reported HPAs alleles' frequency in other ethnicities in the country. This fact highlights the need for a platelet registry to recruit platelet donors from different ethnicities and increase the number of donors by using faster methods.

Keywords: Blood donors; Human platelet antigens; Real-time polymerase chain reaction

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INTRODUCTION

Human platelet antigens (HPAs) are epitopes on the surface of platelet glycoprotein receptors.[1] These polymorphic antigens are bi-allelic systems. The

polymorphic state of HPAs is formed due to a single base-pair substitution in the encoding region, leading to a single amino acid difference in the related glycoprotein.[2]

To date, 41 different platelet antigens have been recognized and are classified in 6 bi-allelic groups as HPAs 1 to 5 and 15 as high-frequency antigens. The low-frequency antigens are HPAs 6-14 and 16-35.[3,4] Different polymorphisms in encoding genes of platelet antigens produce different epitopes that may be recognized by the recipient's immune system during transfusion and trigger immune responses. The most critical complications caused by platelet alloantibodies are platelet transfusion refractoriness (PTR), post-transfusion purpura (PTP), neonatal and fetal autoimmune thrombocytopenia. Studies on the frequency of different HPAs genotypes in different countries have shown that these antigens vary among races and ethnicities.[5-11] These differences between platelet donors and recipients play a critical role in producing alloantibodies against these antigens in multi-transfused patients.[12] Platelet refractoriness is a clinical situation in hematological patients with multi-platelet transfusions.[2,13] Up to 37% of PTR occurs due to HPA incompatibility between platelet donors and recipients.[13,14] To fulfill the purpose of active treatment with platelet products, we need to suffice two requirements: registered platelet-compatible donors and a reliable and fast method for genotyping of platelet antigens.[15] HPAs genotyping has a useful and practical application for managing the reactions and establishing a platelet registry to decrease platelet transfusion reactions.

Historically, polymerase chain reaction with sequence-specific primers (PCR-SSP), and PCR-restriction fragment length polymorphism (PCR-RFLP) were conventional techniques adopted for HPA genotyping. PCR–SSP is the most practical method for HPAs genotyping. The basis of the SSP method is the reduction of Taq-polymerase enzyme efficiency due to the presence of a mismatch at the 3' end between the primers and template DNA. In this method, primers are specially designed for each allele. The nucleotide at the 3' end of the primers, which complements the mutation region, binds to the template DNA only when the sample contains the target allele; therefore, the target DNA is amplified.[2] Despite its simplicity and cost-effectiveness, PCR-SSP is prone to some problems, such as low sensitivity. To obtain high throughput genotyping results for HPA, Real-time PCR techniques, including real-time PCR with hybridization probes, TaqMan probes, high-resolution amplicon melting analysis, and displacing probes, have developed.[16] The TaqMan real-time PCR method utilizes two specific fluorogenic probes for both alleles a and b. These probes anneal to specific positions on DNA. The 5' end of the probe was labeled with a reporter dye Victoria (VIC) and 6-Carboxy-fluorescein (FAM)), and the 3' end with a quencher dye. When the probe is intact to DNA, no fluorescent signal is issued due to the FRET phenomenon. As the PCR continues, the probe bound to the specific sequence is degraded by the 5'-3' DNA Taq-polymerase exonuclease activity, and the reporter dye is released. Each time a PCR cycle is completed, increased fluorescence intensity is observed due to increased products. The development of the minor groove binder (MGB) method allows us to use shorter probes and remove the problems of temperature matching between primers and probes during PCR-SSP.[17-19]

Iran is a country with different ethnicities. To date, studies on HPA have been performed only on blood donors living in Tehran (the capital), and only one study has been performed by ethnicity.[16] As a result, conducting different studies by different ethnicities in Iran is significantly felt. On the other hand, genotyping studies performed in Iran have been done by the PCR-SSP method.

The primary purpose of this study is to determine the allelic and genotype frequency of human platelet antigens in the Azeri ethnicity of Ardabil province. This study is the first study in Iran that aims at HPA genotypes in the Azeri ethnicity. On the other hand, for the first time in Iran, this study used TaqMan Real-time PCR as a method with higher sensitivity and speed to determine platelet antigens' genotype compared with the PCR-SSP method in the Iranian Blood Transfusion Organization (IBTO). The benefits of more accurate and faster methods to determine the HPA genotype are that they can be used in the platelet registry, which is essential for managing platelet refractoriness in any country.

MATERIALS AND METHODS

Samples

This project was approved by the Ethics Committee of the High Institute for Education and Research in

Transfusion Medicine (IBTO) (Approval ID: IR.TMI.REC.1397.009). A total of 100 healthy volunteer donors at the blood transfusion center of Ardabil city (the capital city of Ardabil province in the northwest of Iran) were retrieved. A consent form was signed by each donor before donation and information about the ethnicity of their parents, and grandparents and possible marriage with people from other ethnicity were questioned. Donors with a family marriage with people from other ethnicities were excluded from the study. 5 mL whole blood samples were collected with ethylenediaminetetraacetic acid (EDTA) from each donor. All samples were kept in the refrigerator and transferred to our lab at 2-8°C. Genomic DNA extraction was done by QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with slight modification. The extracted DNA's quality was estimated spectrophotometrically (Nanodrop ND-1000, Thermo Fisher Scientific, and Wilmington DE, USA).

Polymerase Chain Reaction with Sequence Specific Primers (PCR-SSP) and TaqMan Real-time PCR

The primers required for PCR-SSP were adopted from Cavanagh, O.Meyer, and Schuh's studies (Table 1).[17-19] The primers were synthesized by the Sinaclon Company (Tehran-Iran). PCR procedure was designated according to the NIBSC method (https://www.nibsc.org/asset.ashx?assetid=b2dfaa8e-1148-4f73-884f-702162412507, available on 14/09/2019) with slight modification in the temperature profile (Table 2). We used the human growth hormone (HGH) gene as the internal control in the whole HPA evaluation procedure.

PCR cocktail consisted of 1 µL of allele-specific HPA1-5 and -15 "a" and "b" primers (in 12 separate tubes) with 1 µL of common HPA primer (1 µM), 0.5 µL of HGH forward primer, and 0.5 µL of HGH reverse primer (0.25 µM) as internal control, 7.8 µL of RNase and DNase free distilled water, 0.7 µL of DNTP (0.5 mM), 2 µL of PCR 10X buffer, 1.2 µL of MgCl2 (1.5 µM), 2 µL of Betaine (0.5 M); 0.3 µL of Taq DNA-polymerase (Add Taq DNA, ADDBIO INC, South Korea) and 3 µL of DNA (25-50 ng/µL). Nevertheless, hot start Taq DNA polymerase (ADDBIO INC) was added for HPA-3 genotyping. We used McBride's designated primers and probes to set up HPA genotyping by Real-time PCR (Table 3).[20] The TaqMan Real-time PCR assay was performed according to the QuantiTect Probe PCR Handbook's instructions for quantitative, real-time PCR and two-step RT-PCR using sequence-specific probes (Qiagen, Hilden, Germany). The reaction mix containing 0.2 µL forward primer (0.4 µM), 0.2 µL reverse primer (0.4 µM), 0.1 µL allele probe a (VIC) (0.2 µM), 0.1 µL allele probe b (FAM) (0.2 µM), 1.9 µL RNase and DNase free distilled water, and 5 µL 2x QuantiTect Probe PCR Master Mix, 2.5 µL DNA (25-50 ng/µL) was added to give a final volume of 10 µL. The PCR Program for HPA-1 to 5 and 15 consisted of 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60ºC for 20 seconds, and 72ºC for 20 seconds and performed by the Corbet Research Rotor-Gene 3000 (QIAGEN). The data were analyzed by Rotor-Gene 6 and Rotor-Gene Q series software (Qiagen, Hilden, Germany).

A known commercial genotyped DNA (HPA-1 to -5 and -15)(Innotrain, Kronberg, Germany) as external quality control and a validated kit (HPA Ready Gene Innotrain, Kronberg, Germany) for determination of PCR-SSP and Real-time PCR predictive value were used (unpublished data).

Statistical Analysis

$\chi 2$ test was used to evaluate allelic frequencies compatibility to Hardy Weinberg (HW) rule (HWE: aa+bb+2ab=1). To compare allelic and genotype proportion frequencies between Azeris and other populations, the $\chi 2$ test was also used. The significance level was %0.05. Genetic relationships among Azeris and other populations were compared based on the allelic frequency of HPAs 1 to 5 using the Principal Coordinate Analysis (PCoA) method and MVSP software version 3.22 (Kovach Computing Services, Wales, UK; http://www.kovcomp.com/mvsp, available on 09/04/2020).

RESULTS

PCR-SSP and Real-time PCR were used for genotyping 100 blood donor samples with Azeri ethnicity from Ardabil city. The results of PCR-SSP and TaqMan Real-time PCR were 100% consistent with 100% accuracy. The electrophoretic pattern of HPA alleles by the PCR-SSP method has been shown in Figure 1. Also, HPA genotypes frequency and calculated allele frequencies compatible with HWE were shown in Table 4.

Table 1. Primers used for polymerase chain reaction-sequence-specific primer amplification (PCR-SSP).[17-19]

HPA	Primer sequence 5'-3'	Common primer 5'-3'	SNP	Amplicon size (bp)	Ref
1a	TCACAGCGAGGTGAGGCCA	GCAGGTAGAGAGTCGCCATAG	176T	90	18
1b	ACTTACAGGCCCTGCCTCC	AGCCGGAGTGCAATCCTCTG	176C	196	17
2a	GCCCCCAGGGCTCCTGAC	TCAGCATTGTCCTGCAGCCA	482C	258	18
2b	GCCCCCAGGGCTCCTGAT		482T	258	
3a	GGGGGAGGGGCTGGGGA	GGCCCTGGGACTGTGAATG	2621T	293	17
3b	GGGGGAGGGGCTGGGGC		2621G	293	
4a	CTGGCCACCCAGATGCG	GGTAGAAAGGAGCTATAGTTTGGC	506G	253	17
4b	CTGGCCACCCAGATGCA		506A	253	
5a	AGAGTCTAGCTGTTTACTATCAAAGA	CTCTCATGGAAAATGGCAGTACA	1600G	252	18
5b	GAGTCTAGCTGTTTACTATCAAAA		1600A	252	
15a	TTCAAATTCTTGGTAAATCCTGG	ATGAACCTTATGATGACCTATTC	2108C	226	19
15b	TTCAAATTCTTGGTAAATCCTGT		2108A	226	
HGH	Forward GCCTTCCCAACCATTCCCTTA Reverse TCACGGATTTCTGTTGTGTTTC		-	429	18

Table 2. Thermal profiles for human platelet antigens (HPAs) 1 to 5 and 15.

	HPA-1a, 2 and 15			HPA-1b and 4			HPA-3* and 5		
	Temperature	**Time**	**Cycle**	**Temperature**	**Time**	**Cycle**	**Temperature**	**Time**	**Cycle**
Activation	95°C	5 min	1X	95°C	5 min	1X	95°C	5 min	1X
Amplification 1	96°C	25 s	5X	95°C	30 s	10X	96°C	25 s	5X
	68°C	45 s		65°C	1 min		70°C	45 s	
	72°C	30 s		72°C	30 s		72°C	30 s	
Amplification 2	96°C	25 s	20X	95°C	30 s	22X	96°C	25 s	20X
	61°C	45 s		61°C	50 s		61°C	45 s	
	72°C	30 s		72°C	30 s		72°C	30 s	
Amplification 3	96°C	25 s	15X	-	-	-	96°C	25 s	15X
	51°C	1 min		-	-		53°C	1 min	
	72°C	2 min		-	-		72°C	2 min	
Final Extension	-	-	-	72°C	5 min	1X	-	-	-

* For HPA-3 due to the use of the Hot Start enzyme, the activation temperature was 95° C for 10 minutes.

Table 3. Primers for TaqMan real-time polymerase chain reaction (PCR)

HPA	Primer 5'-3'	MGB probe(3' NFQ)
1	Forward CTGATTGCTGGACTTCTCTTTGG	1a 5'(VIC)-CTGCCTC**T**GGGCTC-3'
	Reverse AGCAGATTCTCCTTCAGGTCACA	1b 5'(FAM)-CTGCCTC**C**GGGCTC-3'
2	Forward CTGAAAGGCAATGAGCTGAAGAC	2a 5'(VIC)- CTCCTGA**C**GCCCACA-3'
	Reverse CCAGACTGAGCTTCTCCAGCTT	2b 5'(FAM)- CTCCTGA**T**GCCCACAC-3'
3	Forward TGGGCCTGACCACTCCTTT	3a 5'(VIC)- TGCCCA**T**CCCCAGCC-3'
	Reverse TGATGGGCCGGGTGAA	3b 5'(FAM)- TGCCCA**G**CCCCAGCC -3'
4	Forward CAGAACCTGGGTACCAAGCT	4a 5'(VIC)- CAGATGC**G**AAAGCT -3'
	Reverse CAATCCGCAGGTTACTG	4b 5'(FAM)- CAGATGC**A**AAAGCT -3'
5	Forward GACCTAAAGAAAGAGGAAGGAAGAGTCT	5a 5'(VIC)-TTACTATCAAA**G**AGGTAAAAA-3'
	Reverse ATGCAAGTTAAATTACCAGTACTAAAGCAA	5b 5'(FAM)-TTACTATCAAA**A**AGGTAAAAA-3'
15	Forward TGTATCAGTTCTTGGTTTTGTGATGTT	15a 5'(VIC)- CTTCAGTT**C**CAGGATTT -3'
	Reverse CCAAGAAGTGATAGAATCAGGTACAGTTAC	15b 5'(FAM)- CTTCAGTT**A**CAGGATTT -3'

Table 4. Genotype and allele frequencies of human platelet antigens (HPA) 1 to 5 and 15 in Azeri ethnicity of Ardabil province (n=100)

HPA Genotype	Observed frequency	Expected frequency	Hardy-Weinberg analysis	Allele frequency
1a/1a	0.83	0.837	χ^2 =0.863 *p*-value=0.649	1a= 0.915
1a/1b	0.17	0.155		1b=0.085
1b/1b	0.0	0.007		
2a/2a	0.76	0.774	χ^2 =1.859 *p*-value=0.394	2a =0.880
2a/2b	0.24	0.211		2b =0.120
2b/2b	0.00	0.014		
3a/3a	0.33	0.336	χ^2 =0.069 *p*-value=0.966	3a= 0.580
3a/3b	0.50	0.487		3b =0.420
3b/3b	0.17	0.176		
4a/4a	1.0	1.0	-	4a =1.000
4a/4b	0.0	0.0		4b= 0.000
4b/4b	0.0	0.0		
5a/5a	0.82	0.828	χ^2 =0.613 *p*-value=0.978	5a= 0.910
5a/5b	0.18	0.163		5b =0.090
5b/5b	0.0	0.008		
15a/15a	0.30	0.319	χ^2 =0.611 *p*-value=0.978	15a =0.565
15a/15b	0.53	0.491		15b =0.435
15b/15/b	0.17	0.189		

The allele frequencies were as follow: 91·5% and 8·5% for HPA-1a and -1b; 88% and 12% for HPA-2a and -2b; 58% and 42% for HPA-3a and -3b; 100% for HPA-4a and no HPA-4b was found; 91% and 9% for HPA-5a and -5b, 56.5% and 43.5% for HPA-15a and -15b. There were no homozygosis results for HPA-1b, -2b, -4b, and -5b. The homozygous "aa" alleles were the most frequent genotypes for HPA-1, HPA-2, HPA-4, and HPA-5 and “ab” for HPA-3 and HPA-15.

Two approaches for estimating allelic frequency by TaqMan Real-time PCR were used (quantitative and scatter graph). Figure 2 shows TaqMan real-time

polymerase chain reaction (PCR) analysis of 20 samples for human platelet antigens (HPA)-3. The increased fluorescence intensity of VIC but not FAM indicates homozygous samples for HPA-3aa (figure 2A), and homozygous samples for HPA-3bb are described by the increase in FAM but not VIC (figure 2B). On the other hand, the increased fluorescence intensity of both VIC and FAM indicates heterozygous samples for HPA-3ab (figure 2C). Figure 2D shows the allelic discrimination analysis based on the Scatter Graph. The Y-axis's fluorescent increase indicates HPA-3aa, the X-axis indicates HPA-3bb, and the fluorescent increase in both axes indicates HPA-3ab. The negative control does not show any fluorescent increase in the Y- and X- axis. In both methods, the frequency was compatible and comparable with the PCR-SSP method. PCR-SSP and real-time PCR methods were challenged by the known commercial genotyped DNA (HPA-1 to -5 and -15) (Innotrain, Kronberg, Germany) as the external validated control. Its results were compatible with data issued by manufacturer as follow: HPA-1aa, HPA-2aa, HPA-3bb, HPA-4aa, HPA-5aa, and HPA-15aa. At the same time, DNAs typed by both methods had the same allelic pattern in evaluation by the commercial kit (HPA Ready Gene Innotrain, Kronberg, Germany).

Genetic relationships between populations by the Principal Coordinate Analysis (PCoA) method based on the allelic frequency of HPA-1 to -5 are illustrated in Figure 3. All populations were classified into four categories, and each group was identified by one color (red, blue, black, green, and purple). The Azeri population in the current study was located in the blue group.

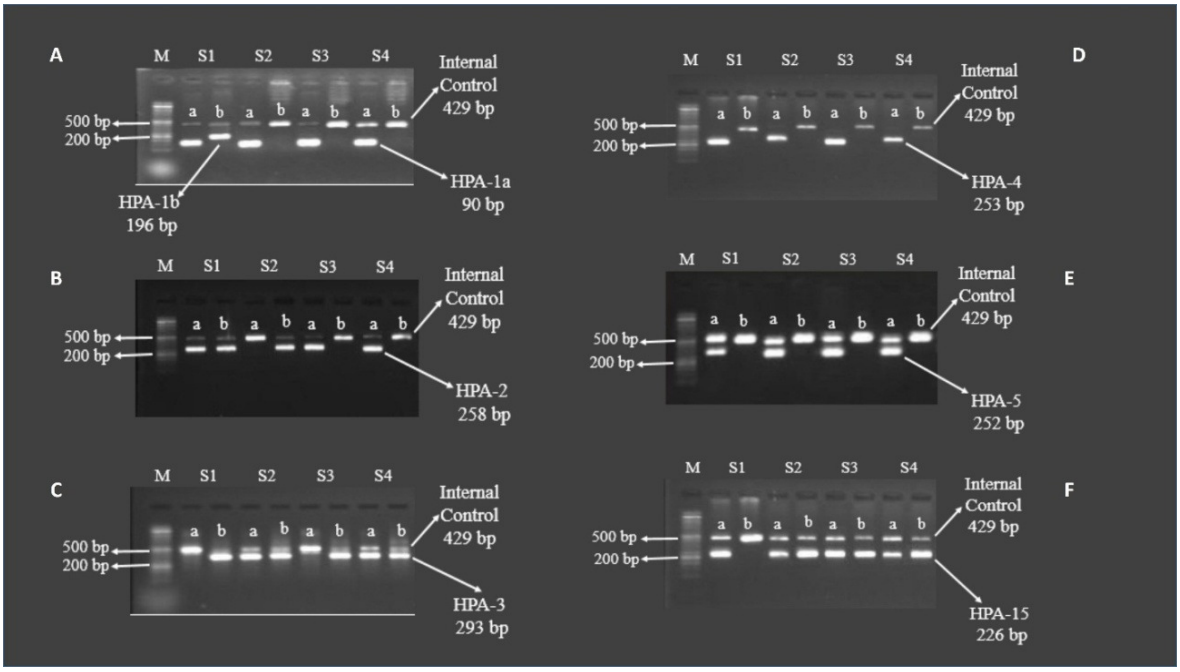

Figure 1. The results of 4 samples for polymerase chain reaction with sequence specific primers (PCR-SSP). S1, Sample 1; S2, Sample 2; S3, Sample 3; S4, Sample 4; M, Size Marker (50 bp); A: human platelet antigens (HPA)-1, B: HPA-2, C: HPA-3, D: HPA-4, E: HPA-5, F: HPA-15

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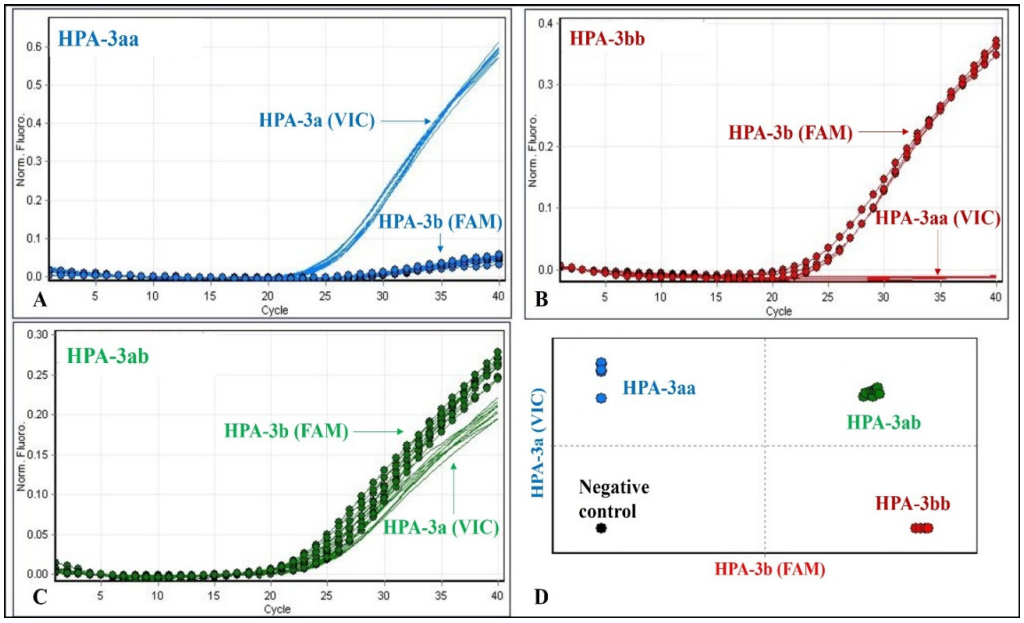

Figure 2. TaqMan real-time polymerase chain reaction (PCR) analysis of 20 samples for human platelet antigens (HPA)-3. All analyzes were performed by Rotor-Gene Q Series software. VIC is specific for allele a, and FAM for allele b. Figures A-C indicate quantitative analysis. The increase in VIC indicates HPA-3aa (A), FAM indicates HPA-3bb (B), and the increase in both, expresses HPA-3ab (C). Figure D shows the allelic discrimination analysis based on the Scatter Graph. The fluorescent increase in the Y-axis indicates HPA-3aa, the X-axis indicates HPA-3bb, and the fluorescent increase in both axes indicates HPA-3ab

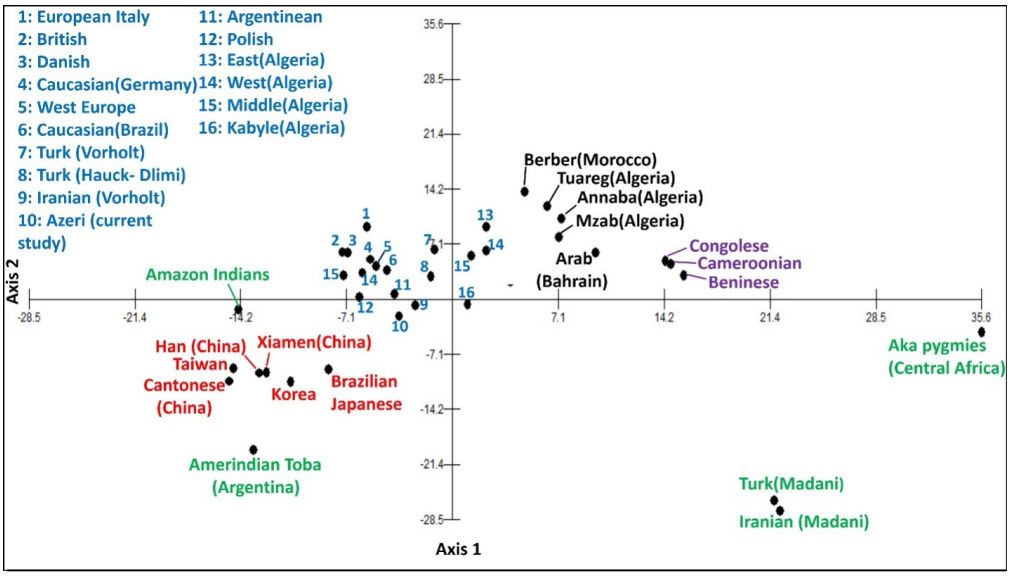

Figure 3. Genetic relationships between various populations based on human platelet antigens (HPA)-1 to -5. European Italy,[12] British,[21] Danish,[22] Caucasian (Germany),[23] West Europe, Turk, Iranian (Vorholt),[15] Caucasian (Brazil),[24]; Turk (Hauck-Dlimi),[25] Argentinean, Amerindian Toba,[26] Polish,[27] East, West, Middle, Kabyle, Tuareg, Annaba, Mzab (Algeria),[28] Amazon Indians,[29] Cantonese,[30] Taiwan,[31] Han (China),[32] Xiamen (China),[33] Korea,[34] Brazilian Japanese,[35] Berber (Morocco),[36] Arab (Bahrain),[37] Congolese, Cameroonian, Beninese, Aka pygmies,[38] Turk (Madani),[39]

DISCUSSION

HPAs have different frequencies among various populations and ethnicities. Allele frequencies of HPA-1 to -5 and -15 in different populations were shown in Table 5.

At present, several methods are being used in many laboratories. In this study, we evaluated allelic frequencies of HPA-1 to -5 and -15 in 100 blood donors with Azeri ethnicity by both PCR-SSP and TaqMan Real-time PCR methods, and both methods had concordant results.

PCR-SSP technique is a widely used method in different laboratories because of its cost-beneficiary and simplicity. However, it has some disadvantages, such as time-consuming post-PCR processes and complexities in interpreting results. The TaqMan real-time PCR, especially MGB probes, makes it possible to genotyping a large number of samples in a faster way.

Ficko genotyped 120 samples for HPAs -1 to -3 and -5 using Real-time PCR and PCR-SSP. Discordant results between both methods, especially in HPA-3, were reported.[40] In contrast, no discrepancy in HPA allelic frequency by both methods was seen in our study. However, we encountered some problems with our PCR-SSP method; for instance, using the recommended primer for HPA-1b by Cavanagh led to many nonspecific electrophoretic patterns; therefore, we replaced the HPA-1b primer set from Meyer's study to improve HPA-1b PCR patterns.[41] Also, by the same approach and changing the thermal profile and quality of Taq-polymerase, we improved the nonspecific pattern of HPA-3. The TaqMan Real-time PCR method, especially MGB format, provides an opportunity for accurate and robust HPA alleles' genotyping. Also, the lack of post-PCR manipulation decreases the contamination and undesirable outcomes.[40,42]

Table 5. Allelic frequencies of human platelet antigens (HPA)-1 to -5 and -15 in different populations

Ethnic origin	Country	HPA1 Alleles%		HPA2 Alleles%		HPA-3 Alleles%		HPA-4 Alleles%		HPA-5 Alleles%		HPA-15 Alleles%		Ref
		1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	15a	15b	
Dutch (1993)	Netherlands	84.6	15.4	93.4	6.6	55.5	44.5	100	-	90.2	9.8	-	-	43
Caucasian (1995)	Austrian	85.2	14.8	91.8	8.2	61.2	38.8	-	-	89.2	10.8	-	-	44
Finns (1995)	Finland	86	14	91	9	59	41	-	-	95	5	-	-	45
Japanese (1995)	Japan	-	-	89.8	10.2	59.4	40.6	99	1	-	-	-	-	46
Japanese (1996)	Japan	99.8	0.2	-	-	-	-	98.9	1.1	-	-	-	-	47
Danish (1995)	Denmark	83.1	16.9	91.7	8.3	62.6	37.4	100	-	92.1	7.9	-	-	22
Caucasian(1996)	Germany	87.9	12.1	86.6	13.4	56.2	43.8	-	-	89.7	10.3	-	-	23
Korean (1998)	Korea	99	1	92	8	55	45	99	1	98	2	-	-	34
Polish (1998)	Poland	87.4	12.6	89.8	10.2	59.2	40.8	100	-	93.7	6.3	-	-	27
Caucasians	Brazil	92.5	7.5	85	15	60	40	100	-	92	8	-	-	7
Black		90.3	9.7	81	19	66.6	33.4	100	-	87.6	12.4	-	-	
Parakana Indians (1999)		100	-	82.1	17.9	75.7	24.3	100	-	100	-	-	-	

Slovenian (1999)	Slovenia	80.9	19.1	89.1	10.9	59.1	40.9	99.7	-	93.4	6.6	-	-	48
Amazon Indians	Brazil	100	-	96.3	3.7	70.8	29.2	100	-	96.3	3.7	-	-	
Brazilian (1999)		91.8	8.2	81.6	13.9	64	36	100	-	82.5	17.5	-	-	29
Berber (2002)	Morocco	74.8	25.2	81.8	18.2	68.2	31.8	100	-	86.1	13.9	-	-	36
Chinese (2002)	Taiwan	99.7	0.3	96	4	57.5	42.5	99.8	0.2	98.5	1.5	-	-	31
British (2003)	UK	84	16	92.5	7.5	62.7	37.3	100	-	91.4	8.6	-	-	21
Xikrin	Brazil													49
Amerindians		-	-	-	-	-	-	-	-	-	-	78	22	
Brazilian (2004)		-	-	-	-	-	-	-	-	-	-	51	49	
Beninese	Benin	89.6	10.4	70.8	29.2	67.9	32.1	100	-	81.8	18.2	64.6	35.4	38
Congolese	Congo	90.4	9.6	77.6	22.4	59.6	40.4	100	-	73.2	26.8	70.1	29.9	
Cameroonians	Cameroon	90.7	9.3	76.3	23.7	61.4	38.6	100	-	74.6	25.4	69.1	30.9	
Aka pygmies (2004)	Central Africa	100	-	60.7	39.3	50	50	100	-	59.5	40.5	69.8	30.2	
Arab (2006)	Bahrain	76	24	76.7	23.3	56.8	43.2	93.2	6.8	86.1	13.2	-	-	37
Iranian (2007)	Iran	98	2	54	46	48	52	100	-	99	1	47	53	39
Xiamen (2007)	China	99	1	93.2	6.7	57.7	42.3	100	-	99	1	59.7	40.3	33
Argentinean	Argentina	87.8	12.2	87.5	12.5	61.2	38.8	100	-	92.7	7.3	51.1	48.9	26
Amerindian Toba (2008)		100	-	94.4	5.6	38.9	61.1	100	-	100	-	68.5	31.5	
Annaba	Algeria	79.3	20.7	81.9	18.1	61.5	38.5	100	-	81	19	-	-	28
East		83.6	16.4	85.2	14.8	66.3	33.7	100	-	84.4	15.6	-	-	
Kabyle		88.7	11.3	82.5	17.5	61.3	38.7	100	-	91.3	8.7	-	-	
Mzab		84.1	15.9	83.5	16.5	59	41	100	-	78.2	21.8	-	-	
Middle		87.3	12.7	85.3	14.7	65	35	100	-	85.9	14.1	-	-	
West		82.3	17.7	85.3	14.7	58.8	41.2	100	-	84.9	15.1	-	-	
Tuareg (2010)		79	21	80.6	19.4	68.8	31.2	100	-	84.5	15.5	-	-	
Cantonese (2010)	China	99.5	0.5	96.2	3.8	54.2	45.8	99.5	0.5	99	1	40.2	59.8	30
Pakistani (2010)	Pakistan	88.5	11.5	92	8	69	31	100	-	90	10	59	41	6

Caucasian	Germany	79.8	20.2	90.8	9.2	56.7	43.2	100	-	91.6	8.4	51.7	48.3	25
Turkish (2012)		86.3	13.7	86.8	13.2	60.7	39.3	99.6	0.4	89.3	10.7	47.4	52.6	
Han (2013)	China	98.8	1.9	94.2	5.8	56	44	99.3	0.7	98.4	1.6	53.7	46.3	32
Caucasian (2015)	Brazil	86.7	13.3	89.5	10.5	62.1	37.9	100	-	90.3	9.7	50	50	24
Brazilian mix	Brazil	88.1	11.9	84.4	15.6	65.9	34.1	100	-	92.5	7.5	52.2	47.8	35
Japanese (2017)		97.8	2.2	89.3	10.7	57.9	42.1	100	-	97.5	2.5	55.3	44.7	
Egyptian (2017)	Egypt	78.7	21.3	86.2	13.8	-	-	-	-	87.5	12.5	-	-	50
European (2018)	Italy	84.7	15.3	94.5	5.5	62	38	99.7	0.3	84.7	15.3	50.3	49.7	12
West Europe	Germany	83.9	16.1	91.2	8.8	57.9	41.9	100	-	89.5	10.5	49.8	50.2	15
Arab		91.3	8.8	87.5	12.5	65	35	100	-	85.4	14.6	50	40.04	
Turk		87.7	12.3	88.6	11.4	64.2	35.8	100	-	85.2	14.8	50.3	9.7	
Iran (2020)		85.7	14.3	87.1	12.9	54.3	42.9	98.6	1.4	91.4	8.6	44.3	55.7	
Azeri Turk (2020)	Iran	91.5	8.5	88	12	58	42	100	-	91	9	56.5	43.5	Current study

Since 2007, we have planned a research project to use different HPAs genotyping methods to find a better technique to establish a platelet registry with platelet donors from different ethnic groups. So we have evaluated HPA-1 to -5 and -15 in various subjects, including 100 blood donors, in women with recurrent abortion and their husbands,[51] in human stem cell donors-recipients,[52] in patients with Hepatitis C virus,[53] in blood donors with Turkman ethnicity,[16] and patients with platelet refractoriness.[54] The current study is also a part of this project.

In our previous study, 100 blood donors living in Tehran, were genotyped for HPA-1, -2, -3, -4, -5 and -15.[39] The allele frequencies were as follow: 98% and 2% for HPA-1a and -1b, 54% and 46% for HPA-2a and -2b, 48% and 52% for HPA-3a and -3b, 100% for HPA-4a, 99% and 1% for HPA-5a and -5b, 47% and 53% for HPA-15a and -15b. We have not found HPA-4b not only in the Azeri population but also in our previous studies. Comparing current results with the above report, showed that there was a significant difference between HPA-2 (p<0.0001) and HPA-5 (p<0.02). Genetic relationships of populations analyzed by the PCoA method showed that Azeri blood donors in the current study were far from blood donors in our previous report.[39]

Nineteen out of 100 blood donors were recorded as Azeris[55] and their HPA alleles were as follow: 98% and 2% for HPA-1a and -1b; 53% and 47% for HPA-2a and -2b; 47% and 53% for HPA-3a and -3b; 100% for HPA-4a and -5a, 44% and 56% for HPA-15a and -15b. No HPA-4b and 5b were found. In our previous study, we recorded blood donors ethnically according to donors' comments, and nothing was questioned about their parents' and grandparents' ethnicity. It should be noted that the number of Azeri blood donors in the previous study was low, and all of them who lived in Tehran had perhaps mixed ethnicities.

In our published data,[52] results of HPAs genotyping by PCR-SSP for HSCT donors-recipients were reported together. But the result of HPAs alleles for 55 HSCT donors were as follows: 1.0 for HPA-1a, 0.545 and 0.455 for HPA-2a and -2b, 0.436 and 0.564 for HPA-3a and 3b, 1.0 and 0.0 for HPA-4a and -4b, 1.0 and 0.0 for HPA-5a and -5b, and 0.481 and 0.519 for HPA-15a and -15b (our unpublished data). There were

significant differences between allelic frequencies of Azeri blood donors and HSCT donors with unknown ethnicities related to HPA-1 (p=0.002), -2 (p<0.001), -3 (p<0.04), and -5 (p<0.002).

Nozarimirarkolaei et al evaluated 80 blood donors of Turkman ethnicity in the north of Iran for HPA-1. Allelic frequency for HPA-1a and -1b was 96% and 4% respectively.[16] The frequency of HPA-1b in our study was higher, but no significant difference was observed between Azeris and Turkmans (p=0.18).

Hauck-Dlimi et al and Vorholt et al studied HPA genotypes in the Turkish population living in Germany.[15,25] Similar to our current study, no homozygousity was found for HPA-1b, -2b, and -4b. There were no significant differences between our current study and Hauck-Dlimi for HPA-1(p=0.24), HPA-2 (p=0.79), HPA-3 (p=0.69), HPA-4 (p=0.52), HPA-5 (p=0.68), and HPA-15 (p=0.19), in general. Although the frequency of the HPA-1b allele was higher in Hauck and Vorholt's study, no significant differences were found between Azeris in current study and Turks in Vorholt report for HPA-1 (p=0.37), HPA-2 (p=0.89), HPA-3 (p=0.36), HPA-5 (p=0.2), HPA-15 (p=0.37). HPA-4a was seen in 100% of both groups.

According to the PCo analysis, Azeris in the current study and Turks living in Germany in Hauck and Vorholt's studies were categorized in the same group. Vorholt evaluated HPAs genotype of 35 donors with Iranian origin but unknown ethnicity as follow: 85.7% and 14.3% for HPA-1a and -1b, 87.1% and 12.9% for HPA-2a and -2b, 54.3% and 42.9% for HPA-3a and 3b, 98.6% and 1.4% for HPA-4a and -4b, 91.4% and 8.6% for HPA-5a and -5b, 44.3% and 55.7% for HPA-15a and -15b. There was no significant difference between Azeris in the current study and the Iranian group in vorholt's study.

Allelic frequencies of HPA-1 to -5 and -15 in different populations worldwide are shown in Table 5. According to the PCoA method, all populations were classified into four categories. The Azeri population is in the second group with more similarity to the European population and far from the Azeri population in our study and the Iranian population in our previous study.[39] A Heterogeneous population with different ethnicities such as Fars (51%) mostly in central regions, Azeris (24%) in the northwest and other parts, Gilaks and Mazandaranis (8%), Turkmen (1%) in the north, Arabs (3%) in south and southwest, Kurds (7%) and Lurs (2%) in the west, and Baloch (2%) in the southeast live in our country. The Turkish-speaking Azeris or Azerbaijanis, also known as Azerbaijani Turks, is the second high ethnic group of the country, mostly living in East and West Azerbaijan, Ardabil, and Zanjan provinces. However, they also live in other parts of the country like Tehran (the capital city),[56] and some have married people from other ethnicities in different cities.

In conclusion, we observed differences in HPAs frequency between blood donors with Azeri ethnicity in the current study and previous study conducted in IBTO on blood donors in Tehran by Madani in 2007 without considering donors' ethnicity. Due to these differences, platelet products' transfusion might lead to alloimmunization and subsequent platelet transfusion reactions. This fact does reflect the need for a platelet registry in the country. Appropriate and reliable, and fast methods for platelet antigens genotyping are crucial in platelet registry centers. These methods should be able to perform throughout the country and be tailored to the economic situation. In this study, real-time PCR was used for the first time in our laboratory to genotype human platelet antigens. This method is much easier and faster than the PCR-SSP. There was no difference between the results of both methods, while the real-time PCR method can be used on a large scale. Platelet genotyping helps identify and manage platelet transfusion reactions, thrombocytopenic alloimmune syndrome, NAITP, and racial studies.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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