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ORIGINAL ARTICLE

Genotyping of Platelet Alloantigens by DNA Sequencing in Pakistani Population

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ABSTRACT

Introduction: Single-nucleotide polymorphism (SNP) in human platelet antigens (HPAs) glycoproteins leads to alloimmunizations and platelet disorders such as posttransfusion purpura, neonatal alloimmune thrombocytopenia, and refractoriness to platelet transfusion. To study the prevalence in a particular ethnic group, genomic DNA is used to genotype HPAs. Detection of these polymorphisms is imperative to identify the risk of alloimmunization and the provision of HPAs. Current study was planned to determine the frequency of HPAs in the Pakistani population of blood donors.

Methodology: Genomic DNA was extracted from blood samples of 300 randomly selected platelet donors from five major cities of Pakistan (Islamabad, Peshawar, Karachi, Quetta, and Mirpur). This study was approved by the ethical committee of Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad, Pakistan. Prior informed consent was taken from all the participants. Sequence-specific primers for platelets glycoprotein genes were designed using Primer 3 online software. The distinct targets were amplified through PCR. Amplified PCR products were eluted from the gel after electrophoresed, purified and sequenced. All the sequences and data obtained were analyzed through SPSS version 25.

Results: Genotyping of samples showed that among the subjected HPA systems, HPA-1, HPA-5, HPA-7w, HPA-19w, and HPA-21w systems were found to have both a and b alleles in the Pakistani population while only aa genotype was found in HPA-4, HPA-6w, HPA-8w, HPA-10w, HPA-11w, HPA-16w, and HPA-23w. The frequency of HPA-1a was 0.9333 and HPA-1b was 0.0666, HPA-5a was 0.8033 and HPA-5b was 0.1966, HPA-7wa was 0.98 and HPA-7wb was 0.02, HPA-19wa was 0.95 and HPA-19wb was 0.05 and HPA-21wa was 0.9866 and HPA-21wb was 0.0133. Among the analyzed HPAs, the mismatch probability was higher in HPA-5 while it was lower in HPA-21w.

Conclusion: HPA-4b, HPA-6b, HPA- 8b, HPA-10b, HPA-11b, HPA-16b and HPA-23b were absent. No homozygosity was found in the remaining genotyped HPAs. Our study suggests that it is necessary to establish HPA screening sites in blood banks to have HPA typed donor registry providing compatible therapeutic platelets to all unimmunized patients. Our data will be useful to understand and better treat the alloimmune-mediated platelet disorders.

Key words: Alloantigens, Genotyping, Sequencing, Platelets, Platelet alloantigens

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[1,2]Conception; Literature research; manuscript design and drafting; [2,3] Critical analysis and manuscript review; [5,6] Data analysis; Manuscript editing.

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Introduction

Platelets are 3-5µm, anucleated cell fragments in blood and critical for hemostasis.[1] Over the last five decades, the transfusion of platelet concentrates is being regularly used for the treatment of thrombocytopenic patients, reducing the incidence and severity of bleeding disorders.[2] The human platelet antigens (HPAs) result from single nucleotide polymorphisms (SNPs) in the genes that encode glycoprotein,[3] expressed on platelet surface membranes, frequently on GPIIb/IIIa.[4] In addition to the HPA, the platelet surface membrane comprises of other antigenic molecules namely ABO blood group antigens,[5] human leukocyte antigens (HLA),[6] and the Nak[a] antigen present on CD36.[7]

HPA polymorphism results in the development of human platelet antibodies following alloimmunization,[8] leading to the destruction of platelets.[9] These alloantibodies develop in response to exposure to the alloantigens after blood transfusion and during pregnancy or transplantation. This may result in platelet disorders including neonatal alloimmune thrombocytopenia (NAIT), posttransfusion purpura (PTP), and refractoriness to platelet transfusion. In NAIT, pregnant women become immunized to alloantigens of fetus platelets, resulting in thrombocytopenia in the infant.[10] The PTP is an infrequently reported adverse reaction of blood transfusion characterized by severe thrombocytopenia within two weeks of transfusion.[11,12] Refractoriness to platelets, on the other hand, occurs when the patient's circulating platelet levels regularly fail to rise after transfusion of an adequate dose of platelets.[13]

Until now, 41 human platelet alloantigens have been identified[14] on six platelet glycoprotein (GP) complexes which are functionally significant; GPIIb, GPIIIa, GPIba, GPIbb, GPIa and CD109.[15] The HPA with a higher frequency is labeled as "a" while with low frequency is labeled as "b". A word "w" attribution is added after the name of antigen if there is no reported alloantibody against antithetical antigen.[5] The genotyping for HPA is required to identify the risk of alloimmunization and the provision of HPA-matched platelets for patients with alloimmune-mediated platelet disorders. HPA typing was formerly performed by serologic techniques.[16] The current methods are based on genomic DNA, such as PCR sequence-based typing,[17] PCR sequence-specific primers,[18] restriction fragment length polymorphism-PCR),[19] real-time PCR,[20] BeadChip Microarray Technology,[21] matrix-assisted laser ionization or desorption time of flight-mass-spectrometry.[22,23] The platelet antigen frequency is different among populations around the globe. Most of the existing data have been reported from North American and Western European regions. However, the incidence of HPA in other regions is still not studied extensively. In particular, data among Pakistani population, are still insufficient and so far, only one study has been published.[24]

This current study was performed to determine the frequency of human platelet antigens (HPA) in Pakistani blood donors to be able to provide HPA-matched platelets for patients with immune platelet disorders.

Methodology

This cross-sectional study was performed from October 2019 to February 2020, at the Department of Pathology and Transfusion Medicine, Pakistan Institute of Medical Sciences (PIMS), Shaheed Zulfiqar Ali Bhutto Medical University (SZABMU), Islamabad, Pakistan. A total of 300 regular healthy blood donors were recruited in this study. These donors were randomly selected following careful selection criteria,[25] from five major cities including Islamabad (PIMS/SZABMU), Peshawar (Regional Blood Centre), Karachi (Regional Blood Centre), Quetta (Regional Blood Centre), and Mirpur

(Department of Pathology and Transfusion Medicine, Divisional Headquarters Teaching Hospital). From each centre, blood samples were collected in EDTA tubes and transported to the study site, following standard protocols of cold chain maintenance. Written informed consent was obtained from all study participants.

This study was approved by the ethical committee of the SZABMU, Islamabad, Pakistan.

Laboratory analyses

DNA extraction

Genomic DNA from the whole blood of all samples was extracted using the inorganic method.[26] The DNA concentration was maintained to 20–50 ng/µl. The extracted DNA was stored at 4ºC for further use. To determine the quality of DNA, the extracted DNA was detected through UV light after performing agarose gel electrophoresis. 2µL DNA was mixed with 1µL bromophenol dye and was loaded onto 1 percent agarose gel. 0.5-gram agarose was dissolved in 50 ml of 0.5x TBE buffer and was placed in a microwave oven until it started boiling. It was then placed at room temperature for cooling. 2 µL ethidium bromide was added and was mixed well. It was poured in gel tray with comb placed in it and placed for solidification at room temperature. After 20 – 30 minutes, comb was removed carefully, and the gel was ready with wells for loading DNA. 2 µL DNA was dissolved with 1 µL bromophenol dye and then each sample of extracted DNA was loaded separately in wells. Electrophoresis was performed for 30 minutes at 90 volts/cm (45 mA). The DNA was then visualized under UV lights by performing gel documentation assay.

Designing sequence-specific primers

HPA polymorphism of nucleotide sequences was obtained from the HPA Immuno Polymorphism Database.[27] Sequence-specific primers were designed (Table 1) according to HPA nucleotide polymorphism and sequence obtained of GP1BA, ITGB3, ITGA2, ITGA2B, CD109 and GP1BB gens in the NCBI GenBank Database using Primer 3 online software.[28] A pair of primers was used to amplify those HPA nucleotide polymorphisms which are located nearer to each other in the same gene. Different primers were designed for different HPA systems. All the designed pairs of primers had a different range of amplification product lengths. The location of primer attachment to the complementary sequence was kept 50 base pairs away from the HPA system polymorphism site.

Table I: Details of primers used for HPA

Name	Primer	Sequence (5'->3')	Tm (°C)	Length Amplicon (bp)	Final concentration
HPA-1 and HPA-10w	Forward	TGCTCCAATGTACGGGGTAA	58.72	424	0.5
	Reverse	TCCCACCCCATTTCCATTCTG	59.99		
HPA-5	Forward	AGACGTGCTCTTGGTAGGTG	59.40	231	0.5
	Reverse	TGGGGACATCCTCAAAAATGA	57.18		
HPA-6w and HPA-7w	Forward	CTGGGCCCAACTGTGTCTAA	59.60	529	0.5
	Reverse	GCAGGTATATGAGGGGGTGTG	59.93		
HPA-4, HPA-16w and HPA-19w	Forward	GGTGGAGGATTACCCTGTGG	59.45	239	0.5
	Reverse	CGTCTGGAGGAGGGACTTAC	58.90		
HPA-8w, HPA-11w, HPA-21w and HPA-23w	Forward	AGCTGGACTGGGATACGCTT	60.98	254	0.5
	Reverse	ACAGGTGGGAGTTGGATAGGT	60.20		

Table II: PCR conditions for HPA-1, -5, -6,-7,-9,-10,-11,-21 and 23						
HPA		Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
HPA-1 and HPA-10w	Temp (℃)	95	95	58	72	72
	Time (Sec)	300	60	60	60	600
	Repeats		35			
HPA-5	Temp (℃)	95	95	59	72	72
	Time (Sec)	180	30	30	60	600
	Repeats		35			
HPA-6w and HPA-7w	Temp (℃)	95	95	61	72	72
	Time (Sec)	180	30	30	60	600
	Repeats		35			
HPA-8w, HAP-11w, HPA-21w and HPA-23w	Temp (℃)	95	95	60	72	72
	Time (Sec)	180	30	30	60	600
	Repeats		35			

Table III: PCR conditions for HPA-4, HPA-16w and HPA-19w									
HPA		Initial Denaturation	Denaturation	Touch down Annealing	Extension	Denaturation	Annealing	Extension	Final Extension
	Temp (℃)	95	95	65	72	95	63	72	72
	Time (Sec)	240	60	60	60	60	60	60	600
	Repeats		15			25			

Amplification of required region using Polymerase Chain Reaction (PCR)

The required amplicon was amplified through PCR using the designed sequence-specific primers. Positive and negative control PCR reactions were also conducted. For this purpose, PCR tubes were used. All the reactions were performed in T100TM Thermal Cycler (Bio-Rad). Mixture for each PCR was made by adding 25 µL master mix (Thermo Genotyping of amplicon (Thermo Scientific), 5 µL forward primer, 5 µL reverse primer, and 5 µL DNA sample. The final volume was set to 50 µL by adding 10 µL PCR water. All these were properly mixed. Different conditions were set to carry out PCR for each pair of primers. Normal PCR was carried out for all pairs of primers (Table 2) except designed for HPA-4, HPA-16w, and HPA-19w, which were amplified through touch-down PCR (Table III)

Amplicons were purified and were sent for genotyping to Macrogen Inc, Korea. After genotyping, all the sequences were analyzed and then blasted with standard sequences of HPA polymorphism reported to Immuno-Polymorphism Database.[27]

Statistical analyses

Statistical analyses were carried out using SPSS version 25.0 (IBM corporation, USA). Tests for Hardy-Weinberg equilibrium in the population were performed by x^2 test. Chi-square test was used to calculate expected and observed. *P* values were assigned statistically significant which were less than 0.05.

Results

Amplicon confirmation

All the PCR products were confirmed by performing gel electrophoresis. Samples were run with a DNA ladder. All the PCR products were randomly loaded to confirm their molecular weight according to the specifically designed primers for PCR. The DNA

ladder used was of 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 from top to bottom respectively. All PCR products had durable and exact single bands (Fig 1).

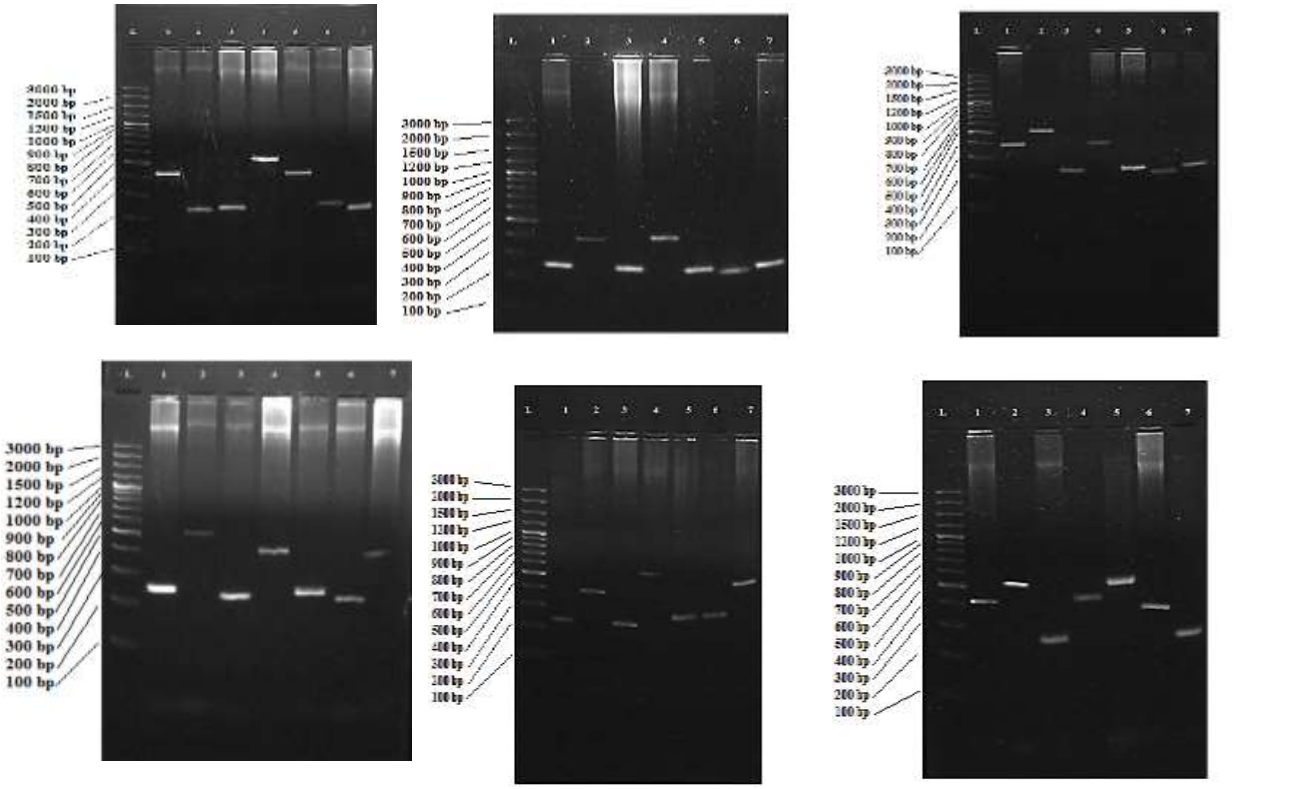

Figure 1: The electropherogram for the HPA system. 231 bp (HPA-5), 239 bp(HPA-4, HPA-16w and HPA-19w), 254 bp (HPA-8w, HPA-11w, HPA-21w and HPA-23w), 424 bp (HPA-1 and HPA-10w) and 529 bp (HPA-6w and HPA-7w) . L: DNA ladder

Genotyping of the amplified region

Genotyping was performed of the PCR products to view the polymorphism of the nucleotide at the polymorphism for each HPA system. Fig 2 shows the sectional sequencing chromatograms of samples. Site of polymorphism is shown in each sequenced segment by an arrow.

The frequencies of HPA-1, 4, 5, 6w, 7w, 8w, 10w, 11w, 16w, 19w, 21w and 23w genotype and alleles in Pakistani population are shown in table 4. Distribution of these HPA genotype loci were compatible with Hardy Weinberg equation (P> 0.05). HPA-4, HPA-6w, HPA-7w, HPA-8w, HPA-10w, HPA-11w, HPA-16w and HPA-23w were perfect aa homozygous while there was polymorphism in HPA-1, HPA-5, HPA-7w, HPA-19w and HPA-21w systems.

HPA mismatch probability

Mismatch probability in Pakistani population were found in HPA-1, HPA-5, HPA-7w, HPA-19w and HPA-21w system. Among those analyzed, HPA-5 showed higher mismatch probability while the least mismatch probability was found HPA-21w. Mismatch probability was low in HPA-1, HPA-7w and HPA-19w system as much of the individuals were aa homozygote.

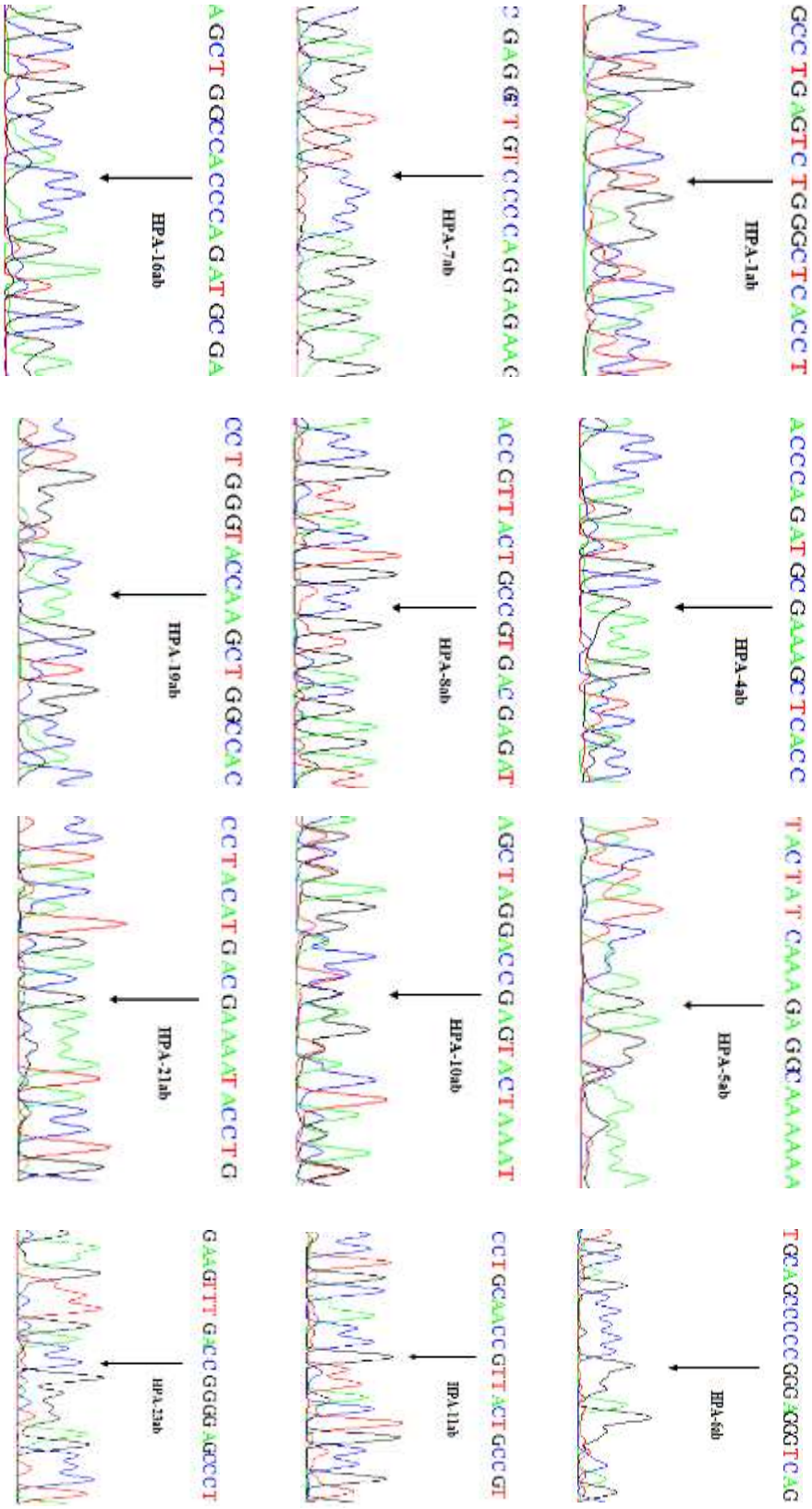

Figure 2: Chromatograms of DNA sequencing for HPA- Frequency

Table IV: Distribution of HPA-1, HPA-5, HPA-7, HPA-19 and HPA-21 in Pakistani population. (n=300)														
HPA	N Observed			N Expected			Hardy-Weinbrg		Genotype frequency			Allele frequency		Mismatch probability
	aa	ab	bb	aa	ab	bb	X^2	*P*	aa	ab	bb	a	b	
HPA-1	261	37	2	260.40	38.19	1.400	0.2952	>0.05	0.8711	0.1244	0.0044	0.9333	0.0666	0.1167
HPA-5	193	95	12	192.80	95.39	11.800	0.0052	>0.05	0.6453	0.3159	0.0386	0.8033	0.1966	0.2660
HPA-7w	288	12	0	288.12	11.76	0.12	0.1249	>0.05	0.9604	0.0392	0.0004	0.98	0.02	0.0384
HPA-19w	270	29	1	269.80	29.39	0.800	0.0551	>0.05	0.9025	0.095	0.0025	0.95	0.05	0.0904
HPA-21w	292	8	0	292.05	7.89	0.053	0.0548	>0.05	0.9735	0.0263	0.0002	0.9866	0.0133	0.0259

Discussion

HPAs are expressed on platelets and also other cells such as monocytes and endothelial cells. Human platelet antigens (HPAs) play a critical role in numerous immune platelet disorders such as NAIT, PTP, and refractoriness. About 10% to 20% of NAIT patients have postnatal intercranial hemorrhage (ICH) with a fatality of 5%. Although patients may have internal organs bleeding, but ICH is the feared cause of death. ICH may have a mortality rate of up to 48%.[29] The reoccurrence of NAIT among consequent siblings having positive platelets antigens is about to 100%.[30] According to National Blood Collection and Utilization Survey (NBCUS), in 2015, 305 cases of PTP were confirmed in the United States. About 30% of PTP patients have major hemorrhage with 10% rate of mortality. Women having children with PTP may have an increased risk of NAIT during their following pregnancy.[31] Clinical studies suggest that 27% to 34% of platelets transfusion have unsatisfactory responses.[32]

The knowledge of antigen frequencies in a population is essential for the medical management of patients with immune-mediated platelet disorders. The pattern of HPA frequency varies in different countries globally. Partial data on HPA frequency in the Pakistani population exist. The current study assessed the gene frequency of HPA for 300 blood donors from five large blood centers in the country. Pakistani people belong to different origins and cultures. Although there are many ethnic groups living in Pakistan, the most known are Panjabis, Pashtuns, Sindhis, Balochis, and Kashmiris. The current study focused on Punjabis, Pashtuns, and Kashmiris.

In Pakistan, the rate of consanguineous marriages is very high.[29-31] Consanguineous marriages cause to have greater chances of many abnormalities which also affect platelets glycoprotein. It may also cause the production of isoantibodies.[32] So platelets glycoprotein is also affected due to consanguineous marriages. DNA-based techniques have become a standard after the discovery that the determination of molecular HPA type is due to the substitution of the amino acid at a specific location. Alleles of HPA systems are different on the basis of SNP except for HPA-14w which is due to the deletion of AAG nucleotide. In this study, we analyzed the frequency distribution of HPA system including, HPA-(1,4,5,6w,7w,8w,10w,11w,16w,19w,21w,23w) using PCR- Sequence-Based Typing Method. All the SNP containing regions of subjected HPA systems were amplified using SSP. Primers were designed to be away more than 50 nucleotides from the site of SNP. Targeted regions were amplified, purified and sequenced. HPA mismatch increases the likelihood

of alloimmunization during pregnancy, allogeneic stem cell transplantation and platelets transfusion. A study from Indonesia revealed that alloimmunization against HPA-1, 2 and 6w is uncommon; whereas HPA-1 is the prevalent alloantigen in caucasians.[33] Our study has reported mismatch probability in HPA-1, 5, 7w, 19w and 21w, which can be a risk for alloimmunization. A study performed in Germany on the Turkish and Caucasian populations, compared the HPA between both groups and observed no statistical differences.[34] Similarly, a study from Brazil showed no statistical difference between the reported alleles frequencies of different ethnic groups.[35] Two more studies compared blood donors and platelet donors of southern Brazil and found statistical significance for HPA-1, HPA-2, HPA-5 and HPA-15.[36,37] A study from Algeria have similarity in alleles frequencies of HPA-4 and 5, but had difference of HPA-1, when compared to our study.[38] The allele frequencies of HPA-1, 4, 8w, 10w, 11w, 16w and 21w in our study were similar to the reported frequencies in Chinese population.[39] There is great difference of HPA-5 genotyping distribution among the population of Pakistan and Iran.[40]

An earlier study from Pakistan[24] reported a Hardy-Weinberg equilibrium deviation towards alleles HPA-3b and HPA-5b, due to increased consanguinity rates. In our study, aa genotypes were observed in all HPA systems except HPA-1, -5,-7w,-19w and HPA-21w. When compared to a previous study,[24] there was a significant difference ($P < 0.05$) in HPA-1a. No significant difference ($P < 0.05$) was seen between the reported and observed HPA-4a and HPA-5a in the Pakistani population. HPA gene frequencies in the population of different countries are shown in Table 5.

Table V: Frequency distribution of HPA system reported in different countries of the world[27]

	Pakistan	Argentina	Austria	Brazil	China	Denmark	Germany	Italy	Spain	Saudi Arabia
HPA-1a	0.9333	0.878	0.852	0.925	0.993	0.83	0.84	0.85	0.81	0.809
HPA-1b	0.0666	0.122	0.148	0.075	0.007	0.17	0.16	0.15	0.19	0.191
HPA-4a	1	1	1	1	0.997	1	-	1	1	0.997
HPA-4b	0	0	0	0	0.003	0	-	0	0	0.003
HPA-5a	0.80	0.927	0.892	0.920	0.991	0.92	0.917	0.9	0.88	0.934
HPA-5b	0.20	0.037	0.108	0.080	0.009	0.08	0.083	0.1	1	0.066
HPA-6a	1	1	1	1	0.980	1	-	-	0	-
HPA-6b	0	0	0	0	0.020	0	-	-	-	-
HPA-7a	0.980	-	-	-	1	-	-	-	-	-
HPA-7b	0.020	-	-	-	0	-	-	-	-	-
HPA-8a	1	-	-	-	1	-	-	-	-	-
HPA-8b	0	-	-	-	0	-	-	-	-	-
HPA-10a	1	-	1	1	1	-	-	-	-	-
HPA-10b	0	-	0	0	0	-	-	-	-	-
HPA-11a	1	-	1	1	1	-	-	-	-	-
HPA-11b	0	-	0	0	0	-	-	-	-	-
HPA-16a	1	-	-	-	1	-	-	-	-	-
HPA-16b	0	-	-	-	0	-	-	-	-	-
HPA-19a	0.950	-	-	-	-	-	-	-	-	-
HPA-19b	0.050	-	-	-	-	-	-	-	-	-
HPA-21a	0.986	-	-	-	0.994	-	-	-	-	-
HPA-21b	0.013	-	-	-	0.006	-	-	-	-	-
HPA-23a	1	-	-	-	-	-	-	-	-	-
HPA-23b	0	-	-	-	-	-	-	-	-	-

In conclusion, it is imperative to have a first-hand knowledge of the frequency of HPA to provide a safe platelet concentrates transfusion to the patients, particularly to those who are refractory or alloimmunized.

Conclusion

This is the first study to genotype HPA systems in blood donors from the five major cities of Pakistan. Our study has reported that HPA-1, -5, -7w, -19w, and HPA-21w systems were found to have both a and b alleles in the Pakistani population while only aa genotype was found in HPA-4, -6w, -8w, -10w, -11w, -16w, and -23w. The study will be important to provide information to prevent and treat alloimmunization triggered by HPA. It will also help to improve blood component therapy. It is necessary to establish HPA screening sites in blood banks to have HPA typed donor registry providing compatible therapeutic platelets to all unimmunized patients. Future research can study possible feasible and effective methods of HPA screening and also provide a guideline for potential HPA screening during pregnancy.

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