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Diego Blood Group Systems Polymorphism in Pakistan

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Submitted for Publication: 21-12-2025
Revision Received: 17-03-2026
Accepted for Publication 18-03-2026

ABSTRACT

Background: Over 300 blood group antigens that belong to 33 blood group systems have been identified, and red blood cell genes demonstrate immunological diversity. The Diego blood group system is clinically significant, and antibodies to its antigens may lead to serious cases of transfusion and hemolytic disease in the fetus and infant. **Objective:** To identify the prevalence of Diego blood group alleles among the Pakistani blood donors with the help of genotype analysis, and to give useful information for future blood transfusion guidelines and plans. **Study Design:** Cross-sectional study. **Settings:** Department of Pathology, Armed Forces Institute of Transfusion (AFIT) and the Hematology Department of AMC Hospital, Rawalpindi Pakistan. **Duration:** One year from January to December 2019. **Methods:** A total of 300 blood donors were selected, and blood DNA was extracted using a commercial kit. After preliminary ABO and Rh D blood typing, Sequence Specific Priming PCR (SSP-PCR) was performed to detect the DI1 and DI2 alleles. **Results:** The allele frequencies for DI1 and DI2 were 0.003 and 0.997, respectively. Genotype frequencies were in Hardy-Weinberg equilibrium, with p-values > 0.05, indicating no significant deviation. **Conclusion:** The findings are useful in the development of a red cell antibody panel, the identification of rare blood donors, and the development of a rare blood donor program. The technique is based on PCR-SSP and can be utilized in a resource-constrained laboratory, which provides a cost-effective and quick way of genotyping blood groups.

Keywords: *Diego, Hemolytic, Genotype, Antigen, Antibody, Polymorphism.*

How to Cite: Jadoon SA, Zahra M, Noor A, Mukhtar T, Iqbal S, Ibad AI. Diego Blood Group Systems Polymorphism in Pakistan. APMC 2026;20(1):14-18. DOI: 10.29054/APMC/2026.1858

INTRODUCTION

Research on red blood cell antigens has grown significantly, particularly since DNA-based methods were developed. The International Society of Blood Transfusion (ISBT) has described more than 300 antigens, and the majority of them belong to one of the 33 blood group systems.[1] Blood group genes are highly polymorphic and exhibit significant immunological importance. The genetic variability within these genes leads to the expression of diverse antigenic determinants on the surface of red blood cells. These antigens can elicit immune responses, particularly during blood transfusion or pregnancy, making them clinically significant in transfusion medicine and immunohematology.[2] Studies have revealed that there are marked variations in the frequency and distribution of erythrocyte antigens among various human populations and ethnic groups.[3] Blood group phenotype can be precisely determined from genomic DNA by understanding the molecular aspects of different blood group polymorphisms.[4,5] Molecular methods for blood group agglutinogens are considered essential in numerous medical conditions, such as antigen typing of chronically transfusion-dependent patients to predict the risk of additional red cell antibodies and to prevent further alloimmunization and transfusion-related complications.[6]

The Diego system consists of dual sets of antithetical antigens (DI1/DI2) and (DI3/DI4) and 18 other low-frequency antigens. Antibodies against DI1 and DI2 have been associated with variable degrees of different hemolytic transfusion reactions (HTRs) and HDFN or erythroblastosis fetalis.[7] Immediate or acute hemolytic transfusion reaction (AHTR) occurs within 24 hours of transfusion. It is a medical emergency associated with rapid hemolysis leading to jaundice, disseminated intravascular coagulation (DIC), and shock.[8]

Hemolytic Disease of the Fetus and Newborn (HDFN) is a disorder characterized by the movement of antibodies from the mother's bloodstream, destroying the fetus's or newborn's red

blood cells. The concerned immunoglobulins could be naturally existing or immune agglutinins, which are produced subsequent to a previous sensitizing event, such as pregnancy or transfusion.[9]

For certain blood group systems, such as the Diego system, specific polyclonal antisera are not readily available.[10] In such situations, identifying red blood cell antigens at the genetic level becomes particularly valuable. Molecular characterization enables large-scale screening of blood donors to detect clinically significant blood group antigens and helps resolve complex serological discrepancies.[11] In principle, any red cell antigen associated with a known DNA polymorphism can be determined through molecular methods, as DNA-based reagents are more consistently available compared to conventional antisera.[12]

The advent of genotyping has significantly expanded our ability to identify erythrocyte antigens beyond the limits of traditional serological techniques.[13] Understanding the genetic basis of blood group alleles allows accurate prediction of blood group phenotypes directly from genomic DNA.[14] As a result, molecular testing is now widely employed for red cell antigen genotyping and has become an essential tool in modern immunohematology practice.[15] DNA-based methods enable the detection of numerous erythrocyte antigens in a rapid, reliable, and reproducible manner, facilitating precise typing of both blood donors and recipients.[16]

Several transfusion centers are implementing a genomic approach to type donors for antigens in addition to ABO and Rh D; that is, extended blood group antigen typing.[17] They are doing so in an attempt to find antigen-negative blood units for those who have the corresponding antibody and also to provide products for prophylactic matching to alleviate.[18] The Diego blood group system has been designated and classified by the International Society of Blood Transfusion (ISBT). This system was named after the first antibody maker in a Venezuelan family during an investigation of HDFN.[19] This research aimed to identify the frequency of alleles of the Diego blood group system (DI1 and DI2) among Pakistani blood donors using molecular genotyping. The purpose of this research was to give valuable information on the distribution of Diego antigens in the Pakistani population, which is essential in enhancing the application of transfusion medicine, identification of rare blood donors, and determination of incompatibilities that cause haemolytic transfusion reactions and erythroblastosis fetalis. The primary objective of this study was to investigate the molecular distribution of blood group antigens, with particular emphasis on the Diego blood group system, in the Pakistani population, where such data are currently limited. Although it has been reported in previous literature that blood groups are distributed across ethnicities, there is not much information about the genetic distribution of blood groups in the diverse ethnic groups in Pakistan. The knowledge of these frequencies is crucial in the case of efficient transfusion management, and the PCR-SSP technique employed in this study provides a cost-effective and viable method of conducting an ordinary blood group genotyping, particularly in limited resource bases.

METHODS

The present study was carried out at the Armed Forces Institute of Transfusion (AFIT) and the Hematology Department of Army Medical College, Rawalpindi. The interval of study was one year, that is, from January to December 2019. The sample size calculated using the World Health Organization (WHO) calculator was 184. However, 300 samples were incorporated in this study to get a better representation of our population. Sampling method: non-probability purposive sampling. Samples were collected in accordance with the distribution of ABO blood types in Pakistan. This was done to get a sample that is an actual representative of the study population. Polymorphism is described as the occurrence of a gene in two or more than two alternate forms within a group of people, where each allele is found in at least 1% of the population.[20] Hemolytic transfusion reactions (HTRs): These are reactions in which red blood cells are destroyed due to the recipient of a blood transfusion.[21]

This study was approved by the Institutional Review Board (IRB) and the Ethical Review Committee. Permission was taken from the National Institute of Medical Sciences (NUMS). Informed consent was taken from all the participants. Healthy and unrelated blood donors reporting to AFIT were selected irrespective of gender and ethnicity. Every person included in this study had their relevant medical history obtained in accordance with the National Health Service's questionnaire.

The ABO blood group system is unique in that there is an inverse relationship between the antigens present on red blood cells and the naturally occurring antibodies found in the serum. This procedure is a centered system, centered on the principle that the antigen-positive red blood corpuscles will clump in the company of the existing positive agglutinins directed against the agglutinogen.

RhD testing is done to decide if the D antigen is exist, agglutinizing exists, or if agglutination is lacking, on the red blood corpuscles. Clumping of red cells by means of Anti-D, exists or is anti-D anti-D antisera, which shows that the RBCs possess D antigen and is taken as a positive test result. Whereas the absence of anti-D absence of clumping is interpreted as a negative outcome and shows that the D antigen isn't discernible. Genomic DNA was extracted from whole blood using a commercially available DNA extraction kit (Gentra Puregene Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA was subsequently subjected to polymerase chain reaction (PCR) to amplify a specific DNA region containing the target nucleotides of interest. PCR amplification involves three main steps: denaturation of the DNA double helix, annealing of primers to the complementary sequences, and extension of the target DNA strand by DNA polymerase. Blood group agglutinogens of the Diego classification systems were selected, and the PCR-SSP techniques were used to determine the genotypes. PCR-SSP was established to identify the following alleles, DI1/DI2, DI1/DI2, of the SLC4A1 gene. The sequence of primers, their orientation, melting temperatures, and final concentration are given in the table 1.

Table 1: **Primer sequence and parameters for PCR**

Blood Group System or gene	Final concentration (µmol/l)	Tm	Orientation	Sequence	Allele
Diego	0.25	57.7	Forward	GGGCCAGGGAGGCCA	DI1 DI2
	0.25	60.4	Forward	GGGCCAGGGAGGCCG	
	0.25	59.2	Common Rev	CCTGCCAGCTCCATGTGAC	
HGH	0.1	57.3	Control	GCCTTCCCAACCATTCCCTTA	
	0.1	53.6	Control	TCACGGATTTCTGTTGTGTTT	

In gel electrophoresis, charged molecules like DNA, RNA, and proteins can be separated with the help of an electric current administered to a gel matrix. Due to this negative charge, they move from the negative to the positive electrode on applying electric current. The migration of the molecules through the gel corresponds to their molecular weight.

Agarose gel electrophoresis allows the separation of molecules with large differences in size, whereas polyacrylamide gel electrophoresis is suitable for unravelling DNA fragments with trivial differences in their sizes.

Statistical analysis and allele frequency calculation:

Using Microsoft Excel spreadsheets, the genotypic and the allelic frequencies of the Dombrock, Diego, Colton, and Scianna blood systems were directly counted. Allele/gene frequencies were calculated using the following formula: Allele frequency = number of alleles / 2 (sample size). The genotype frequencies of these blood groups were established by using the Hardy-Weinberg equation, which states that p2+2pq+q2=1, where 'p' signifies the occurrence of the dominant allele in a population and 'q' is the occurrence of the recessive allele within the same population. P2 is the fraction of the homozygous dominant persons; q2 denotes the frequency of the homozygous recessive persons, whereas 2pq is the fraction of the heterozygous persons. Also, the sum of allele frequencies, that is, 'p' plus 'q', should be equal to one. The chi-square tests were used to compare the expected and observed genotype frequencies (χ2 test). The formula is:

$$\chi^2 = (\text{observed} - \text{expected})^2 / \text{expected}$$

The chi-square test was applied to assess differences in antigen frequency distribution among various ethnic groups within Pakistan, as well as between the Pakistani population and other global populations. A p-value of less than 0.05 was considered statistically significant, while a p-value greater than 0.05 was regarded as not statistically significant.

RESULTS

Three hundred unrelated and healthy blood donors between the ages of 18 and 63 (29.9 ± 7.6) were included in this study. The remaining donors were all male (99.7%), with only one female (0.3%). Of the 300 donors, 35 (11.6%) had the blood type AB, 90 (30%) had the blood type B, 92 (30.7%) had the blood type O, and 83 (20.7%) had the blood type A. Regarding their Rh status, 263 (87.7%) were Rh positive, whereas 37 (12.3%) were Rh negative, as shown in the table 2.

Table 2: The ABO and D blood groups' distribution in the blood donors

ABO/Serology	Number of samples and Percentage
O	30.7% (n=92)
A	27.7% (n=83)
B	30% (n=90)
AB	11.6% (n=35)
Rh D (Positive)	87.7% (n=263)
Rh D (Negative)	12.3% (n=37)

The analysis of the ethnic structures of the study subjects showed that the largest proportion of the respondents comprised the Punjabis (59.3 percent), ahead of the Pathans (23 percent). Minor percentages were Kashmiri (6%), Other ethnicities, which comprised Hazarawals, Balti, and Mohajirs (7%), Sindhi (4%), and Balochi (0.7) table 3.

Table 3: Distribution of major ethnic groups of the individuals incorporated in this study

Ethnicity	Percentage (%) and Frequency (n)
Punjabi	59.3 (n=178)
Pathan	23.0 (n=69)
Sindhi	4.0 (n=12)
Balochi	0.7 (n=2)
Kashmiri	6.0 (n=18)
Other (Hazarawals, Balti, Mohajirs)	7.0 (n=21)

The allele frequency analysis of the Diego blood group system in the Pakistani people indicated that the genotype frequencies were mostly DI2/DI2, which was present in 99.7% of the cases, with only 0.3% of the DI1/DI1 genotype. There were no heterozygous DI1/DI2 genotypes. The allele frequency of DI1 was discovered at 0.003, and the allele frequency of DI2 was 0.997. The genotypic equilibrium was in Hardy-Weinberg since the p-value exceeded 0.05 ($p > 0.05$), and it will hence no longer deviate significantly by any means shown in the table 4.

Table 4: Allele frequencies of Diego systems in the Pakistani population: Hardy-Weinberg equilibrium status

Blood group	Genotype	Allele frequency	Expected value	Observed value	χ^2	p
Diego	DI1/DI1	0.003	0.0009	0.3	0	>0.05
	DI2/DI2	0.997	99.4	99.7		
	DI1/DI2		0.598	0.00		

The degree of ethnic variation was evident in the allele frequencies of the Diego blood group system (DI1 and DI2) in the various ethnic groups of Pakistan. The alleles of DI1 were less common in the Punjabi population, with D11 (DI) having a frequency of 0.006, and the DI2 allele (D12) as the dominant allele, having a frequency of 0.994. Conversely, the DI1 allele was not found in the Pathan, Sindhi, Balochi, and Kashmiri, and other ethnic groups; the DI2 allele was found in all individuals with a frequency of 1.000. This finding outlines the fact that the DI1 allele is comparatively uncommon and is confined to the Punjabi ethnic group, whereas DI2 is dominant in all other ethnic groups of Pakistan, as shown in the table 5.

Table 5: Allele frequencies of Diego blood groups among different ethnic groups in Pakistan

Blood group	Diego
Allele	D11 D12
Frequency in Punjabis	0.006 0.994
Frequency in Pathans	0.000 1.000
Frequency in Sindhis	0.000 1.000
Frequency in Balochis	0.000 1.000
Frequency in Kashmiris	0.000 1.000
Frequency in others	0.000 1.000

DISCUSSION

We performed a survey at the molecular level to determine the allele frequencies of Diego systems. It is crucial for the blood banks to have the knowledge of blood group antigen frequency distribution in their population to carry out routine transfusion services, to resolve blood group discrepancies, and to supply compatible blood products to the patients. Many studies have been conducted at the molecular level to determine the blood group antigen frequencies of Caucasians and Blacks, but little or no data is present concerning the distribution of various blood group agglutinogens in Pakistan. The Diego system encompasses various antigens; of these, DI1 and DI2 are the most important. Incompatibilities of the Diego antigens have been stated to cause isoimmunisation in the foetus and neonate and haemolytic transfusion reactions. DI2 is rampant in most parts of the world, while DI1 is less frequent. Occurrence of the DI1 antigen shows ethnic variation. It is considered a Mongoloid attribute. It is more prevalent in Koreans (10.5%), Japanese (7.9%), and Chinese (4.4%). A study reported the frequency of the DI1 antigen to be equal to 5% in the Thai population.[21,22]

In a study from South Gujarat, India, the frequency of DI1 and DI2 was found to be 1% and 99%, respectively.[23] The occurrence of DI2 ranges from 90 to 100% in most parts of the world, being 99.7% in Pakistan. The knowledge of the pervasiveness of the various red blood corpuscle blood group agglutinogens in different regions and ethnicities is valuable in transfusion medicine. This is since the donor-recipient incompatibilities at the antigenic level lead to the production of antibodies,[19] which are responsible for life-threatening outcomes such as haemolytic transfusion reactions and immunization in the foetus and neonate. Several studies show that the distribution of blood type antigens varies significantly between geologically and archaeologically distinct groups. Therefore, determination of the occurrence of various blood group antigens at a sub-population level can serve as a source of beneficial data for transfusion services,[20] particularly in a multi-ethnic country like Pakistan. Such information can be used to search for potential donors. Serological techniques are costly, and many antisera are not commercially available.[22] Erythrocyte blood group allele genotyping can help to screen donors for the common as well as the rare blood group antigens.[23]

The allele frequency analysis of the Diego blood group system in the Pakistani population revealed a predominance of the DI2/DI2 genotype (99.7%), with a very low frequency of the DI1/DI1 genotype (0.3%) and no observed DI1/DI2 heterozygous individuals. This distribution appears unusual because, under Hardy–Weinberg equilibrium, the presence of two homozygous genotypes would normally be accompanied by heterozygous individuals. However, this finding may be explained by the extremely low frequency of the DI1 (Di^a) allele, which is known to be rare in many populations, while the DI2 (Di^b) antigen is a high-frequency antigen worldwide. Previous studies in Asian and Middle Eastern populations have similarly reported a strong predominance of the DI2 allele and very low frequencies of DI1, supporting the pattern observed in this study. The absence of heterozygotes may also reflect factors such as limited sample size or population structure, and further studies with larger sample populations are recommended to confirm these findings (Figueroa, 2013; Nathalang *et al*., 2016; Wu *et al*., 2010).[24-26]

Several considerations are made while choosing an appropriate molecular approach, including cost, test duration, sensitivity and specificity, efficiency, and equipment availability. Several high-throughput DNA platforms are commercially available.[27] Consequently, we chose the PCR-SSP method for this study. Sequence-specific priming PCR (PCR-SSP) is simple, specific, and a comparatively inexpensive molecular technique. It can be used in routine practice for red blood cell blood group genotyping and can be easily implemented in developing countries.

CONCLUSION

Our study provides the first data on the Diego blood group polymorphism in Pakistan. The data from the present report can help in developing precise red cell antibody screening/identification panels in accordance with the blood group polymorphism in the local population. Diego's blood groups should be considered medically significant for the reason that antibodies directed against their antigens can result in both types of haemolytic transfusion reactions as well as erythroblastosis fetalis. The present report is significant for transfusion services in our country. Furthermore, since Pakistani people have extensively migrated all over the world, this data will have international implications.

LIMITATIONS

This study had certain limitations. The sample size was relatively limited and may not fully represent the genetic diversity of the entire Pakistani population. Additionally, participants were recruited from specific regions, which may limit the generalizability of the findings to all ethnic groups in Pakistan.

SUGGESTIONS / RECOMMENDATIONS

Further large-scale studies involving diverse ethnic groups across different regions of Pakistan are recommended to better understand the distribution of the Diego blood group system.

Such studies may also help improve blood transfusion safety by developing more comprehensive red cell antibody screening and identification panels based on local antigen polymorphisms.

CONFLICT OF INTEREST / DISCLOSURE

The authors declare no conflict of interest.

FUNDING SOURCE

Not available.

ACKNOWLEDGEMENTS

The authors would like to acknowledge all the participants who voluntarily took part in this study and the staff members who assisted in sample collection and laboratory procedures.

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