



Original Article



Allelic Distribution of the Colton Blood Group System in Pakistani Blood Donors

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ABSTRACT

More than 300 blood group antigens from 33 separate blood group systems have been characterized, many of which are highly immunogenic. The blood group genes are highly polymorphic among various populations. Colton blood group antigens, for example, are clinically important, and the presence of antibodies against them can cause hemolytic disease of the fetus and newborn and hemolytic transfusion reactions. **Objectives:** To find the allelic distribution and the genotype frequencies of C01 and C02 of the Colton blood group system among the Pakistani blood donors. **Methods:** A cross-sectional study at the Armed Forces Institute of Transfusion, Rawalpindi, took place from March 2020 to March 2021 with ethical approval. The study selected 300 blood donors and extracted DNA from their EDTA-anticoagulated blood using a kit. After determining ABO and Rh D groups, C01 and C02 alleles were identified via Sequence Specific Priming PCR. The study analyzed the amplified products by polyacrylamide gel electrophoresis, calculated gene frequencies, and performed statistical analysis using the chi-square test. **Results:** The C01 and C02 allele frequencies were 0.997 and 0.003, respectively. The most common was C01/C01 (99.7%), and C02/C02 (0.3%), and no C01/C02 heterozygous was found. There was no indication of a non-uniform distribution of genotypes ($\chi^2 = 0.36$, $p > 0.05$) in relation to Hardy-Weinberg equilibrium. **Conclusion:** The C01 allele can be found widely in the Pakistani population, and through this, the pattern of the Colton blood group allele and the practice of transfusion in Pakistan can be explained.

INTRODUCTION

Since the advent of DNA-based methods, the study of red blood cell antigens has become a thriving field of study. 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 polymorphic and immunogenic [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, 4]. 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 [5]. Further immunization, patients having red cell autoantibodies and a positive direct Coombs test, patients receiving multiple transfusion therapies, to arrange antigen-negative blood when polyclonal sera are unavailable, and to ascertain the possibility of developing hemolytic disease of the fetus and newborn (HDFN) in prenatal medicine [6]. Colton system



currently contains four antigens; of these, the high prevalence antigens are CO1 and CO4, while CO2 and CO3 are not common [7]. Immediate or acute hemolytic transfusion reaction (AHTR) occurs within 24 hours of transfusion. It is a medical emergency and is associated with rapid hemolysis leading to jaundice, disseminated intravascular coagulation (DIC), and shock [8]. While delayed hemolytic transfusion reaction occurs within 24 hours up to 28 days post-transfusion, and is characterized by profound anemia with a hemoglobin level often below the pre-transfusion value [9]. HDFN is a disorder characterized by the trans- placental movement of antibodies from the mother's bloodstream, destroying the fetus's or newborn's red blood cells. HDFN may lead to hyperbilirubinemia or anemia or both, which affects the fetal or neonatal morbidity as well as mortality [10]. Theoretically, all red cell antigens with known DNA polymorphisms can be typed using molecular techniques because, unlike antisera, reagents for DNA-based typing are available in abundance [11]. With genotyping, we can identify far more erythrocyte antigens than were previously possible with serological testing alone [12]. Using DNA-based methods, it is possible to determine the presence of numerous erythrocyte agglutinogens more accurately [13].

The study presents the first data on the allelic distribution of the Colton blood group system in Pakistani blood donors; however, the study was a single-center study, had a relatively small number of participants, and was almost entirely of males, resulting in the findings being less representative of the diverse Pakistani population. However, limited molecular data on the distribution of Colton blood group alleles in the population are available in Pakistan, and it is also not possible to establish comprehensive donor registries or give antigen-matched blood for safe transfusion practices. This study aimed to identify the prevalence of genotyping methods for Colton blood group system polymorphisms among Pakistani

people. To be precise, the study aimed at estimating the frequency of alleles and genotypes of CO1 and CO2 alleles in healthy blood donors.

METHODS

The cross-sectional study was carried out at the Armed Forces Institute of Transfusion (AFIT) and the at the Department of Hematology, Army Medical College, Rawalpindi from March 2020 to March 2021 with ethical approval (ERC/ID/21 dated 9th March, 2020). All the participants signed an informed consent before they could be included in the study as per the Declaration of Helsinki and the institutional ethical rules, developing a non-probability sampling plan sequentially to ensure the viable representativeness of the donor population. The sample size was determined with the help of the WHO sample size calculator, with an expected prevalence of 50 %, a confidence level of 95 %, and a margin of error of 5%, which resulted in the minimum required sample size of 184. Nevertheless, 300 samples were used to enhance the statistical power and to get an improved representation of the study population [14]. The criteria used for inclusion were healthy, voluntary blood donors between the ages of 18 and 65 years. Whole blood was collected under aseptic conditions (approximately 35ml of peripheral venous blood) using sterile disposable syringes and transferred to EDTA-anticoagulated tubes using a commercially available DNA extraction kit (Gentra Puregene Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was then extracted and kept at -20oC pending analysis. Colton blood group alleles (CO1 and CO2) were genotyped by a method known as Sequence-Specific Priming Polymerase Chain Reaction (PCR-SSP). The amplification reaction consisted of denaturation, annealing, and extension reactions, according to standard procedures reported in earlier studies [15]. Polyacrylamide gel electrophoresis was performed on the PCR products (Table 1).

Table 1: Primer Sequence and Parameters for PCR

Blood Group System or Gene	Allele	Orientation	Sequence	Tm	Final Concentration (μmol/l)
Colton	CO1	Forward	GAACAACCAGACGGC	49.5	0.15
	CO2	Forward	GAACAACCAGACGGT	54.4	0.15
High	—	Common Rev	GTTTCTTGGAGCAGGTTAAACA	46.8	0.15
		Control	GCCTTCCCAACCATTCCCTTA	57.3	0.1
		Control	TCACGGATTCTGTTGTGTTT	53.6	0.1

A 6% polyacrylamide gel electrophoresis (PAGE) was used for the separation of PCR products with small size variations. Acrylamide and N, N'-methylenebisacrylamide were used to prepare the gel, and the reaction was initiated with ammonium persulfate (APS) and N, N', N'-tetramethylethylenediamine (TEMED). Once the gel was polymerized, PCR products were mixed with loading dye and loaded into the wells along with a DNA molecular weight marker. The gel was run at 150-200 V for 20-45 minutes in 1× Tris-borate-EDTA (TBE) buffer. The gel was silver-stained after electrophoresis, and the amplified DNA was detected under ultraviolet (UV) light. Band sizes were compared to the molecular weight marker. The predicted size of

the products of C01 and C02 alleles was (insert bp sizes here), and bands were viewed in such a manner. Using Microsoft Excel spreadsheets, the genotypic and allelic frequencies of the Colton blood systems were directly counted. For the calculation of allele/gene frequencies, the following formula was used: allele frequency = number of alleles / 2(sample size). The genotype frequencies of these blood groups were calculated using the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$), where p represents the frequency of the dominant allele and q represents the frequency of the recessive allele in the population. The term p^2 corresponds to the proportion of homozygous dominant individuals, q^2 represents the proportion of homozygous recessive individuals, and $2pq$ indicates the proportion of heterozygous individuals. The sum of the allele frequencies ($p + q$) equals one.

Observed genotype frequencies were compared with the expected frequencies using the chi-square (χ^2) test. The formula is: $\chi^2 = (\text{observed} - \text{expected})^2 / \text{expected}$. The frequency of alleles in various ethnic groups was analyzed descriptively. The p-value was designated as significant when its value was less than 0.05 and not significant if the p-value was more than 0.05.

RESULTS

Among the 300 blood donors, blood group O was the most common at 92 (30.7%), followed closely by group B at 90 (30%), group A at 83 (27.7%), and AB at 35 (11.6%). Regarding RhD status, the majority of donors were Rh positive at 263 (87.7%), while 37 (12.3%) were Rh negative (Table 2).

The genotype frequency of the Colton blood group system was dominated by C01/C01 (99.7 percent) with a very small frequency of C02/C02 (0.3 percent) and no heterozygous C01/C02. The percentage abundance of alleles was estimated at 0.997 of C01 and 0.003 of C02. The distribution of the observed genotypes was in agreement with Hardy-Weinberg equilibrium, and the difference was not statistically significant ($\chi^2 = 0.006$, $p > 0.05$). C01 had an allele frequency of 0.997 (95% CI: 0.991-1.000), and C02 had an allele frequency of 0.003 (95% CI: 0.000-0.009). It was 0.997 (95% CI: 0.991-1.000), 0.000 (95% CI: 0.000-0.012), and 0.003 (95% CI: 0.000-0.009), respectively (Table 4).

Table 4: Observed and Expected Genotype Frequencies of the Colton Blood Group System in Pakistani Blood Donors (n=300) Showing Hardy-Weinberg Equilibrium

Blood Group	Gen-type	Observed Frequency (n)	Expected Frequency	95% CI	Allele Frequency	χ^2	p-value
Colton	C01/C01	0.997 (299)	0.994	0.991-1.0	p = 0.997	0.006	>0.05
	C01/C02	0.000 (0)	0.006	0.000-0.012	q = 0.003		
	C02/C02	0.003 (1)	0.00001	0.000-0.009	—		

The distribution of genotype frequencies of all analyzed genes was in Hardy-Weinberg equilibrium, without significant differences in the observed and expected values of the frequencies (p -value > 0.05). The alleles of the Colton blood group (C01 and C02) were found to be distributed in different ethnic groups in Pakistan, with the C01 allele being dominant in all groups. The Punjabi, Pathan, Sindhi, Balochi, and Kashmiri groups showed a C01 allele frequency of 1.000 and a C02 allele frequency of 0.000, with all the groups showing full preponderance of the C01 allele. Other ethnic groups (Hazarawals, Balti, and Mohajirs) had a slightly lower C01 frequency of 0.952 and a C02 frequency of 0.048, implying the low representation of the C02 allele in this subgroup (Table 5).

Table 2: The ABO Blood Groups' Distribution among the Blood Donors

ABO/Serology	n (%)
O	92 (30.7%)
A	83 (27.7%)
B	90 (30%)
AB	35 (11.6%)
D+	263 (87.7%)
D-	37 (12.3%)

The ethnic distribution of the participants of the study revealed that the majority of the study subjects were the Punjabi group with 178 (59.30%), then the Pathan with 69 (23.00%). Kashmiri and Other ethnic groups (including Hazarawals, Balti, and Mohajirs) represented an equal proportion of 18 (6.00%) and 21 (7.00%) of the participants, respectively. Smaller percentages were found to be among the Sindhi group (12, 4.00%) and the Balochi group, 23 (7.6%) (Table 3).

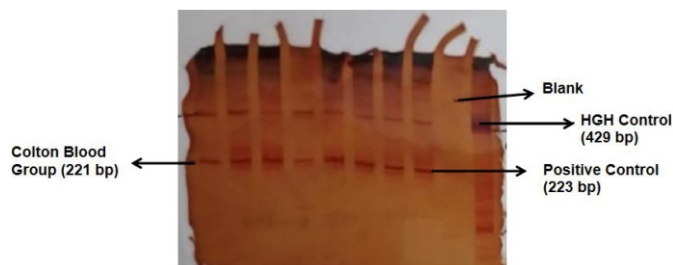
Table 3: Distribution of Major Ethnic Groups of the Individuals Incorporated in This Study

Ethnicity	n (%)
Punjabi	178 (59.30%)
Pathan	69 (23.00%)
Sindhi	12 (4.00%)
Balochi	23 (7.6%)
Kashmiri	18 (6.00%)
Others (Hazarawals, Balti, Mohajirs)	21 (7.00%)

Table 5: Allele Frequencies of Colton Blood Groups among Different Ethnic Groups in Pakistan

Blood Group	Colton
Allele	C01
	C02
Frequency in Punjabis	1.000
	0.000
Frequency in Pathans	1.000
	0.000
Frequency in Sindhis	1.000
	0.000
Frequency in Balochis	1.000
	0.000
Frequency in Kashmiri	1.000
	0.000
Frequency in Others	0.952
	0.048

The study shows a polyacrylamide gel electrophoresis (PAGE) result used for multiplex PCR-based red blood cell (RBC) genotyping, specifically for the Colton blood group systems. The gel appears as a vertical slab with multiple lanes containing DNA bands. On the left side, the lanes are labeled as "Blank" (no DNA control) and "Positive Control". On the right side, annotations indicate specific targets or controls, including "HGH control" and "C01." Distinct horizontal bands are visible across several lanes, representing amplified DNA fragments of different sizes corresponding to specific blood group antigens. The presence and position of these bands indicate successful amplification and genotyping of the respective alleles. The overall image demonstrates the separation of PCR products based on size, confirming the detection of C01 gene variants. The gel electrophoresis image demonstrates successful separation of the PCR amplicons according to their expected product sizes. The target C01 amplicon was detected at 221 base pairs (bp), while the positive control produced a band at 223 bp, confirming the validity of the amplification process. The human growth hormone (HGH) internal control yielded a band at 429 bp, verifying the integrity and adequacy of the extracted DNA. Overall, the presence of bands at the expected sizes confirms successful amplification and detection of the C01 gene variants (Figure 1).

**Figure 1:** Multiplex PCR RBC Genotyping for C01: Polyacrylamide Gel Electrophoresis

DISCUSSION

The current study performed a survey at the molecular level to determine the allele frequencies of Colton systems. It is crucial for the blood banks to have 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 molecular-level studies have examined blood group antigen frequencies in Caucasian and Black populations; however, little to no data are available on the distribution of blood group agglutinogens in Pakistan. This study is the first to report the prevalence of the C01 and C02 alleles in the Pakistani population. One of the greatest weaknesses of this research is the very skewed gender balance of the sample, as 99.7% of the respondents were men. This limits the applicability of the results to the population of Pakistan in general, especially to possible gender-specific variations in the Colton blood group allele frequencies. The agglutinogens of the Colton classification system are positioned on Aquaporin 1 (AQP-1). Anti- C01 and Anti- C02 are clinically important as they can cause hemolysis of the fetus and newborn red blood corpuscles as well as hemolytic transfusion reactions. C01 is the high prevalence antigen, whereas C02 is its low frequency counterpart. C01 is ubiquitous, and its frequency ranges from 98 to 100% in most populations around the world [16, 17]. This research result is in line with those of earlier studies, which show that the C01 allele is very common, whereas C02 is scarce in most of the populations on Earth. There is, however, very little information available on the distribution of the Colton blood group alleles in the South Asian communities, including Pakistan. Thus, it is difficult to compare it directly with the studies of the region [18]. Molecular testing methods offer an accurate mode of predicting phenotype from genotype. Compared to classical hemagglutination-based methods, molecular techniques have many advantages [19]. Genotyping of erythrocyte blood group alleles can be used to screen donors for both common and rare blood group antigens, increasing the chances of providing antigen-matched blood products for patients who require frequent transfusions [20]. In addition, red blood cells with unique antigen profiles can be used to develop antibody identification panels [21]. Moreover, DNA-based methods achieve quicker and higher throughput at a lower cost [22]. Mass-scale screening of blood donors can be implemented in transfusion centers by using molecular approaches [23].

This study has numerous limitations, even though it has some benefits. Since the sample size is small and a huge proportion of male donors were used, the findings might not be representative of the entire population in Pakistan. Moreover, this was a single-center study, and the prevalence of rare alleles like the Colton blood group system (C02) might not have been adequately reflected.

Besides, the PCR-SSP method was used in isolation, and more advanced molecular methods, such as next-generation sequencing, could provide greater genetic data. Nevertheless, additional studies are required to analyze the Colton blood group polymorphisms distribution in Pakistan in a larger and multi-centre cohort of more heterogeneous demographic groups and by the best method of genotyping.

CONCLUSIONS

As it is shown in this study, the C01 allele is quite widespread, whereas the C02 allele is quite uncommon among Pakistani blood donors. The findings present the baseline data of the allelic distribution of the Colton blood group system that could lead to better practice of donor matching and transfusion in Pakistan.

Authors' Contribution

Conceptualization: SAJ

Methodology: SAJ, DEN, SNK

Formal analysis: MTHK, NS, DEN, SNK

Writing and Drafting: SAJ, MTHK, NS, MZ, DEN

Review and Editing: SAJ, MTHK, NS, MZ, DEN, SNK

All authors approved the final manuscript and take responsibility for the integrity of the work

Conflicts of Interest

All the authors declare no conflict of interest.

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