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Serological and Molecular Characterization of Blood Group A2 in Pakistan

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ABSTRACT

Objective: To determine the frequency of Blood Group A2 genotype among Group A Pakistani whole blood donors.
Study Design: Cross-sectional study.
Place and Duration of Study: Armed Forces Institute of Transfusion (AFIT), Rawalpindi Pakistan, from Jan 2019 to Jan 2020.
Methodology: One thousand (1000) healthy and unrelated blood donors were selected. The blood samples were typed for ABO, and those of Blood Groups-A and AB were further subtyped with the help of anti-A1 lectin to categorize them as A1, non-A1, A1B and non-A1B Groups. Next, DNA of non-A1 samples was extracted, and a Polymerase Chain Reaction using sequence-specific primers (PCR-SSP) for type A2 was performed, followed by polyacrylamide gel electrophoresis (PAGE).
Results: Among one thousand blood donors, 247(24.7%) were typed as Blood Group-A, 94(9.4%) as AB-Group, 339(33.9%) B-Group and 320(32%) O Group with variable strength of reaction with ABO antisera. A and AB Blood Groups were further sub-grouped as A1 202(20.2%), A1B 77(7.7%), non-A1 45(4.5%) and non-A1B 17(1.7%). Anti-A1 antibodies were detected in 6(13.3%) of non-A1 samples. PCR of non-A1 samples showed 32(12.9%) to be genotypically A2, and the remaining 13(5.2%) were not A2 and were not further resolved.
Conclusion: Blood Group A2 is not a rare Subgroup in our population. PCR-SSP is a more specific technique than anti-A1 lectin for establishing the Blood Group status of an individual.

Keywords: A2 blood group, Anti-A1 lectin, Polymerase chain reaction using sequence specific primers (PCR-SSP), Serology.

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INTRODUCTION

Transfusion medicine took a revolutionary turn when Karl Landsteiner discovered the ABO Blood Group system in the early 20th century.[1] The gene, which leads to the expression of ABO antigens, is located on chromosome number 9 on position 9q34.q34.[2] Four major antigens are included in the ABO Blood Group system; A, A1, B and AB antigens.[3] ABO locus has three alternate forms; A and B are co dominant, i.e., these will be expressed whenever the gene is present, and O is a silent recessive allele expressed when neither A nor B is present.[2] Blood Group A is frequent in most populations and can be categorized as A1, A2 and other weak A Subgroups.[4] In 1911, von Dungern and Hirschfeld categorized Blood Group A into A1 and A2 as the prime Subgroups of Blood Group A.[5,6]

Serological testing was considered a gold standard technique for blood grouping.[7] Less antigenic load, recent blood transfusions and diseases (autoimmune haemolytic anaemia and aplastic anaemia) are some cases in which phenotype cannot be determined accurately and are major limitations of serological testing.[8,9] A2 and weak variants of Blood Group A gave similar reactions on serological testing with anti-A1 lectin. Therefore, more sensitive and specific DNA-based testing is needed to differentiate.[7] Simpler molecular techniques are now available, which ensure accurate testing in cases of such discrepant results.[10] The prevalence of Blood Group A2 varies in different populations, races and places. Therefore, there needs to be data available on the A2 genotype in Pakistan. With this background in mind, we conducted this study to determine the A2 genotype among healthy blood donors in Pakistan.

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METHODOLOGY

The cross-sectional study was conducted at the Armed Forces Institute of Transfusion (AFIT) Rawalpindi Pakistan, from January 2019-January 2020. The sampling technique used in this study was convenient non-probability sampling. Approval was taken from the ethical review board (ERC/ID/17). Informed con-sent was taken from the participants. The sample size was calculated using the WHO sample size calculator, taking a confidence level of 95%, a margin of error of 5%, and the reported prevalence of

the A2 Blood Group of 20%. The estimated sample size came out to be 247 blood donors.[11]

Inclusion Criteria: The blood donors, who met the criteria of healthy blood donors, were included in our study irrespective of Blood Groups.

Exclusion Criteria: Blood donors who did not give consent, did not fulfil the criteria of healthy donors, or were blood relatives of other blood donors were excluded from this study.

Their information and data were confidential and unavailable to anyone outside the team. Every participant was given a code number. ABO and Rh forward typing was performed on EDTA-preserved whole blood by tube method using blood grouping reagents (Lorne Laboratories) according to the manufacturer's instructions. In addition, reverse typing with commercially available A, B and O screening cells (Biorad) was performed. All samples with Blood Groups A and AB were further subtyped serologically with commercial Anti-A1 lectin (Lorne Laboratories) and labelled as A1, Non-A1, A1B and Non-A1 B Blood Groups.

Genomic DNA was extracted from the EDTA-preserved whole blood non-A1 sample using the QIAamp DNA blood kit (Qiagen, Hilden) according to the manufacturer's instructions. The sequence of the A2 gene was selected as described by Gassner and confir-med from the NCBI website.[12] The pair of primers used in most studies were chosen for our study. As shown below, specific primers of A2 were used for the amplification sequence.

A2-sense primer (5′→3′): GAG GCG GTC CGG AAG C

A2-antisense (5′→3′): GGG TGT GAT TTG AGG TGG GGA C

HGH-sense (5′→3′): GCCTTCCCAACCATTCCCTTA

HGH-antisense (5′→3′): TCACGGATTTCTGTTGTGT TTC

Human Growth Hormone (HGH) was used in each PCR reaction mixture as the internal control. The concentration of sequence specific primer was 2.5pmol (picomols), while that of HGH control primer was 0.25pmols. The volume for one reaction mixture was 17.5µl, which consisted of 15µl PCR buffer, 1.5µl genomic DNA and 1µl of sequence specific primer. The PCR reaction mixture was Dream Taq Green Master Mix (2x) of Thermo Scientific Cat No.K1081. Dream Taq Green PCR master mix contains Dream Taq DNA polymerase, 2x Dream Taq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4mM each, and 4mM $MgCl_2$. Control samples were prepared, which contained HGH primers, DNA & buffer, whereas blank samples contained only sequence-specific primers and buffer.

Amplification of gene products of interest in the PCR mixture was done in a thermocycler (2720 Applied Biosystem) with conditions of initial denaturation at 95˚C for 120 seconds; 5 incubation cycles for 30 seconds at 95˚C, 150 seconds at 59˚C and 15 seconds at 72˚C; 30 incubation cycles at 95˚C for 30 seconds, 61˚C for 60 seconds and 72˚C for 15 seconds and 2 minutes at 72˚C.

Gel Electrophoresis was carried out on a vertical SDS-PAGE gel system of BIO-RAD consisting of 6% polyacrylamide gel and was run at 200V for 30 minutes. The Gel was stained with 0.1% silver nitrate ($AgNO_3$) and counterstained with a mixture of 1.5% sodium Hydroxide (NaOH) and 37% formaldehyde (100ml NaOH+100µl formalin). After the appearance of visible bands, the Gel was washed and transferred to a filter paper. Figure-1 shows gel electro-phoresis of PCR products using sequence specific primer pair.

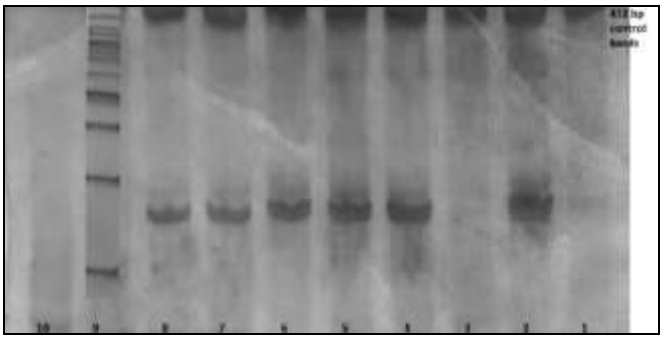

Figure: Gel Electrophoresis of PCR Products Obtained Using the Sequence-Specific Primer Pairs. Positive Bands of 169 Base Pairs (BP) Can be Seen in Lane Number 2 and 4 to 8. Lane number 1 and 3 were Negative Showing Only 432 bp Internal HGH Control Bands. Lane 9 was 100bp Ladder and Lane 10 was Reagent Blank

Collected data were analyzed by Statistical Package for the social sciences (SPSS) version 22:00. Quantitative variables were expressed as Mean±SD and qualitative variables were expressed as frequency and percentages.

RESULTS

A total of 1000 healthy and unrelated volunteer blood donors were included in the study. The mean age was 28±6 years ranging from 18 to 52 years. Approximately 99% of the participants were identified as men and 01% as women. Ethnically 164(66.4%) were Punjabi, 49(19.8%) were Pashtuns, 7(2.8%) were Sindhi, 4(1.6%) were Saraiki, 16(6.5%) Kashmiri, 6(2.4%) Hazarawal and 1(0.4%) Balochi.

Among one thousand blood donors, 247(24.7%) were typed as Blood Group A, 94(9.4%) AB, 339(33.9%) B and 320(32%) O with variable strength of reaction with ABO antisera (Table-I). A and AB blood groups were further sub-grouped as A1 202(20.2%), A1B 77 (7.7%), non-A1 45(4.5%) and non-A1B 17(1.7%). Table-II showed the result of PCR-SSP among non-A1 samples. Among forty-five blood donors, 32(12.9%) were genotypically A2, and the remaining 13(5.2%) were not A2. Gel electrophoresis of PCR products were obtained using the sequence-specific primer pairs as shown in the Figure.

Table-I: Frequency of ABO Blood Groups on Serology (n=247)

Blood Group-A		Blood Group-O	Blood Group-B	Blood Group-AB	
Blood Group-A1	Blood Group-Non-A1			Blood Group-A1B	Blood Group-Non-A1B
n(%)	n(%)	n(%)	n(%)	n(%)	n(%)
202(20.2)	45(4.5)	320(32)	339(33.9)	77 (7.7)	17 (1.7)

Table-II: Results of PCR-SSP among Non-A1 blood Samples (n=247)

Genotyping	n(%)
Positive A2 bands	32(71.1)
Negative A2 bands	13(28.8)

DISCUSSION

This was the first study of its kind in Pakistan on the magnitude of the Blood Group A2 genotype. Two hundred and forty-seven (24.7%) individuals were found to have Blood Group A. Among 247 individuals, the frequency of Blood Group A1 and non-A1 was 202(81.7%) and 45(18.2%) on serology, respectively. The frequency of Blood Group A1 and A2 genotypes were 81.7% and 12.9%, respectively. The remaining 5.2% of the individuals were suspected of having subgroups other than A1 and A2. Overall frequencies of A1 and A2 calculated among all ABO Blood Groups are 20.2 % and 3.2%, respectively.

A study conducted in northern Pakistan has documented similar results showing the frequency of Blood Groups A1 and A2 being 21.6% and 3.2%.[13] The prevalence of Blood Group A1 and A2 in Rayalaseema (India) is approximately 95.8% and 4.1% with anti-A1 lectin, respectively.[14] study from west India has described similar results showing the prevalence of A2 on anti-A1 typing to be around 3.0%.[15] Another study from south India showed the frequency of A1 and A2 on serology to be 98.1 % and 1.8%, respectively.[16] Again, these results are in clear contrast to our study.

The frequency of A1 and A2 in the Chinese population is 99.4% and 0.6%, respectively.[17] Consistent results are obtained in Japan, where the frequency of A1, A2 and other variants is estimated to be around 99.7%, 0.09% and 0.12%, respectively.[18] Our present study findings are closer to the studies conducted on Caucasians, showing the prevalence of A1 and A2 to be 80% and 20%, respectively.[17]

The prevalence of the A2 genotype has also been described in some studies. In a study conducted in China, 88% of serologically A2-positive samples were classified as A2-related alleles on DNA sequence analysis.[18] Frequency of A2 genotyping using a multiplex PCR–RFLP method in Kuwaiti donors was 8.7%

Approximately 99% of all the Blood Group A individuals are categorized as A1 and A2, and the remaining 1% are weak variants, e.g., A3, Ax, Ael and Am subgroups. A3 is the commonest Blood Group among all the weak variants of Blood Group A, with an estimated frequency of 1:1,000 in India and 1:90,000 in Canada. The incidence of Ax and Aend (1:14,448 and 1:43,344, respectively) in the Indian donor population is higher than that estimated in French donors (1: 40,000–1:77,000 & 1:75,000, respectively).[20] These subgroups give discrepant results on serology and special tests, e.g., serum glycosyltransferase estimation and genotyping are required to confirm these subgroups.[21]

Being the first study in Pakistan describing the prevalence of A2 based on genotype, it is recommended that reverse blood typing be included in the routine blood ABO typing so that the individuals of Blood Group non-A1 with anti-A1 antibodies should not be missed. Moreover, routine testing with anti-A1 lectin should be considered compulsory. When an individual is labelled as Blood Group A2, he should be informed, and special cards mentioning his exact Blood Group status should be part of a transfusion protocol. Once the recipient/patient is identified with A2 antigen and anti-A1 antibodies, he should be transfused with A2 or O whole blood or red cells.[22] Discordance between serology and genotyping of A2 Subgroups has been observed in our study. Non-A1 and non-A2 Groups found on genotyping require further studies to characterize the same subgroups.

LIMITATIONS OF STUDY

The potential was the inability to further characterize the other variants of Blood Group A on genotyping because of budgetary constraints.

CONCLUSION

Blood Group A2 is not a distinctive Subgroup in our population. The vast majority of non-A1 Blood Groups were genotypically confirmed as A2 (71.1%) but 28.8% were neither A1 nor A2, i.e., other weak variants of Blood Group A. Samples which were not resolved on genotyping could be other known A Group variants or a novel mutation in the A gene. Further characterization with molecular methods and allele frequency studies are needed for better characterization and understanding of its clinical significance.

Conflict of Interest: None

Author's Contribution

IH & NS: Study design, data interpretation, critical review, approval of the final version to be published.

NUD & MSY: Conception, drafting the manuscript, approval of the final version to be published.

SAK & MAN: Data acquisition, data analysis, critical review, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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