Frequency Distribution Of *ABO* Alleles In A Malaria-Endemic Area Of Pakistan

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Research Article

Keywords: Malaria, ABO, molecular characterization, genotypes, ABO alleles.

Posted Date: February 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1347088/v1

Abstract

Background: Falciparum malaria has exerted adaptive responses in mankind over a period of years for their survival advantage. Red cell disorders like thalassemia, Glucose 6 phosphate dehydrogenase deficiency, sickle cell anemia parallel the endemicity of malaria and are associated with malaria resistance. Distribution of ABO types had been linked to the prevalence of malaria. Pakistan has a huge burden of malaria, and the commonly reported blood phenotype is O. Despite this, no local information is available for allelic frequency of blood types. The study aimed in determining the frequency of *ABO* blood group alleles in a malaria-endemic area of Pakistan.

Methods: A total of 90 blood samples from healthy blood donors residing in Southern Pakistan were included in this study. Serological testing was performed using gel column agglutination and the conventional tube method. DNA extraction was performed from whole blood using Extra-gene I kit) followed by molecular genotyping by polymerase chain reaction, using sequence-specific primers (SSP-PCR) BAGene ABO kits.

Results: Of 90 samples, O phenotype constituted 33.3% (n=30) of the total ABO types. Twelve genotypes were identified in a combination of 6 common *ABO* alleles. Allele frequency of *ABO*O.01.01* was highest at 0.4944 followed by *ABO*B.01* (allelic frequency of 0.2555) and *ABO*A1.01* (allelic frequency 0.1666).

Conclusion: This study demonstrated the *ABO*O.01.01* as the most frequent *ABO* allele in a malaria-endemic area at Pakistan and may be considered as an adaptive response to malaria. We need large scale studies for determining *ABO* alleles in different regions of the country depending on variability in malaria transmission.

Background

World malaria report showed 241 million cases of malaria in 2020 with an estimated deaths of 627000 patients globally [1]. Infection with *P. falciparum* contributes to 90% of malaria mortality and remains the most significant public health challenge worldwide [2]. Historically, this deadly nature of malaria resulted in several adaptive responses in humans for their survival advantage. For example, geographical distribution of many red cell disorders like sickle cell disease, thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency parallel with the endemicity of malaria and have been associated with malaria resistance[3]. Furthermore, there is some evidence that the selective pressure exerted by malaria was responsible for global distribution of ABO blood types [4]. Studies have shown that falciparum malaria is frequent in sub-Saharan Africa where blood type is predominantly O preventing severe infection [5] while residents of colder climate are mostly blood type A where malaria is not endemic [4]. Compared to non-O blood type, decrease resetting of plasmodium, selective oxidative injury and enhanced phagocytosis of parasitized O red cells may be protective against falciparum malaria [6].

Clinically, ABO is the most important blood group system due to its high immunogenicity among the thirty three other blood group systems [7]. Karl Landsteiner in 1900 was the first to describe the agglutination of

red cells antigens with the antibodies present in the sera of different individuals and named these as A, B, O and AB. The ABO locus is located on the distal end of the long arm of human chromosome 9 [8] [9] at the position of 9q34.1-q34.2 and consists of 7 exons (28 to 688 base pairs) and 6 introns (554 to 12982 base pairs) with the last two exons encoding for the catalytic domain of ABO glycosyltransferases mediating the expression of A and B antigens [10]. The ABO locus has three alternative allelic forms, A, B, and O known as *ABO*A1.01* (or A1), *ABO*B.01*, and *ABO*O.01.01* (or O1) respectively. The A allele encodes **α-1,3-N-acetylgalactosamine transferase** that adds N-acetyl galactosamine to the H antigen forming the A antigen while B allele encodes for **α-1,3-**galactosyltransferase which transfers galactose to the H antigen to assemble the B antigen [11]. The H antigen (acceptor substrate) remains the same for both transferases with differences in the donor sugar substrate whereas the O allele does not produce an active enzyme. Figure 1 shows the distribution of *ABO* alleles in the context of malaria distribution according to world malaria report by World Health Organization (WHO) [1].

Situated in South Asia, Pakistan has a huge malaria burden with 60% of its 205 million population having a high risk for malaria. The two prevalent species are *Plasmodium vivax* and *Plasmodium falciparum* causing 88% and 12 % of malaria infection respectively [12]. Though vivax malaria is endemic and seen throughout the year in most places, an alarming high prevalence was noted in falciparum malaria for many provinces in Pakistan [12]. Our previous work showed the presence of G6PD deficiency in a malaria-endemic region [13] while blood type O was reported as the most frequent (ranging from 28.8% to 35.5%) ABO type in the country [14-16]. Despite, these facts, no local data is available for *ABO*-allelic frequency. Karachi, the most populated city in Pakistan observes a high transmission of malaria through the year [17] and is an attractive model for *ABO* genotyping. This study aimed in molecular characterization of ABO blood groups to determine the frequency of *A, B, and O* alleles in a malaria-endemic city at Southern Pakistan.

Materials And Methods

Setting:

This study was conducted at the blood bank, Aga Khan University Hospital (AKUH) during July 2020 till June 2021. The blood bank was accredited with College of American Pathologists in 2016 and observes some 25 to 30,000 blood donors annually. It performs various immunohematology tests for donors and recipients and support thalassemia, hematology/oncology patients as well as bone marrow transplants and surgical procedures at the hospital. Every healthy blood donor is verbally screened for malaria infection. Those having symptoms of malaria or having a history of malaria infection are excluded from donation. Each donor is tested for malaria infection through immuno-chromatographic (ICT) membrane assay (BinaxNOW, Malaria, Abbott, USA) that uses monoclonal antibodies to detect *P. falciparum* and pan-malarial antigen. Peripheral smear review is performed routinely for positive malarial ICT results.

Sample collection and transportation:

A sample size of 90 was calculated with a confidence interval of 95% and 10% margin of error. Frequency of blood phenotypes A, B, O and AB have been reported respectively as 0.23, 0.35, 0.33 and 0.09 by our blood bank in a previous study [18]. Based on this information, consecutive healthy blood donors were enrolled, and the sampling for a particular blood type was concluded when the desired number was reached for that blood type. After completion of routine ABO phenotyping, EDTA blood samples were transported and archived in research molecular laboratory, Aga Khan Medical College for further testing.

Serological testing:

Briefly, routine forward and reverse ABO grouping was performed using gel column agglutination cards (DiaClon ABD / DiaClon ABO/D +/ ID-Card NACL, Diahem diagnostic products, Switzerland) as per manufacturer instructions on automated platform (BioRad, IH1000). For quality control, DiaMed Basics QC kit was used. Conventional tube method was used for resolving ABO discrepant results in gel methodology.

DNA extraction:

DNA extraction was performed from whole blood (Extra-gene I kit #7059, BAG Healthcare, Germany) according to manufacturer's instructions and isolated DNA samples were stored frozen at -80°C till further analysis.

Molecular genotyping (SSP-PCR):

Purified leucocytic DNA was used for ABO genotyping through Sequence-Specific Primers (SSP)-PCR (ABO-type kit# 6640 /ABO-type variant kit #6641, BAG Diagnostics, Germany). The samples were first tested with ABO-type kit that contained eight pre-aliquoted dried down reaction mixtures having alleles and control specific primers and nucleotides for ABO alleles (O^1, O^2 A^1, A^2, and B^1). Those samples where genotyping remained unresolved were tested on ABO-type variant kit containing 18 pre-aliquoted reaction mixtures for various ABO alleles (O^1, O^2 A^1, A^2, A^3, A^x, A^{el}, A^w, and B, B^3, B^x, B^w, and cis-ABO). A ladder (100 bp) was used as a pre-determined standardize marker for determining the size of unknown DNA products and human growth hormone (500bp) was utilized as an internal positive control. Amplification was optimized to a final volume of 10 µl, containing aqua dust, 10x PCR buffer, DNA solution (50-100 ng/µl), and Happy Taq (#70976, BAG Diagnostics, Germany) according to manufacturer's protocol. Each well was loaded with 10 µl amplification mixture (total of 8 wells/sample in ABO-type and 18 wells/sample in ABO-type variant kit). Gel electrophoresis (2% agarose gel) followed by ethidium bromide (EtBr) staining was utilized for separation of PCR products. Results were documented and interpreted on the worksheets provided with the kits. According to the DNA product size, alleles were classified into $O0^1$ (134bp), non-$O0^1$ (140bp), $O0^3$ (194bp), non-$O0^3$ (193bp), B^101^1 (195bp), non-B^101^1 (194bp), A^201^1 (172bp), non-A^201^1 (173bp) in the ABO-type kit. Additional workup with ABO-variant kit identified following ten alleles: *O02* (240bp), A^x (170bp), B^3 (170bp), A^{el}/A^w (238bp/148bp), A^w (234bp), A^w (235bp), A^3/B^x (173bp), B^w (181bp), O^v (167bp) and *cis-AB* (140bp) along with the eight alleles as described for ABO-type kit. ABO

alleles and variants were named as per nomenclature provided by the International Society of Blood Transfusion (ISBT).

Statistical analysis

Data was entered in excel sheet and analyzed for determining the frequencies of various blood groups. For determining allelic frequency, the number of specific alleles was divided by the total number of alleles. Comparison of *O* alleles with *A*- and *B*- alleles was made by dividing the sum of different *O* alleles with that of *A* and *B* respectively. Such ratio was calculated for this study and compared with that of available data for various malaria-free and -endemic countries. WHO world report of malaria was used as a reference to define endemicity of malaria.

Results

We analyzed a total of 180 ABO alleles from 90 apparently healthy individuals residing in a malaria-endemic area of Pakistan. ICT malaria test was negative in all the donors. Table 1 shows the phenotype-genotype distribution among the study participants. Phenotype O was observed in one third of the study participants.

Molecular genotyping results

Molecular studies showed 12 common genotypes among the participants. Of the 21 samples with A phenotype, 18/21 (85.7%) were heterozygous (Fig.2A) while 3/21 (14.3%) were homozygous for A allele with *O.01.01/A1.01* (O^1A^1) as the most common genotype. *O.01.01/A2.01*genotype was identified in A2 (A sub) phenotype (Fig. 2B). Similarly, in samples with B phenotype, 24/31 (77.4%) were heterozygous (Fig. 2C**)** while 7/31 (22.6%) were homozygous for B allele and *O.01.01/B.01* ($O^1B^{1)}$ was the most frequent genotype. In O phenotype, 22 /30 individuals (73.3%) were homozygotes for *O.01.01/O.01.01* (Fig.2D) while *O.01.01/O.02.01* and *O.01.01/O.01.02* were less frequent genotypes. Eight samples with AB phenotype were genotyped as *A1.01/B.01* (Fig. 2E).

Allele distribution

The frequency distribution of ABO alleles is summarized in Table 2 which shows that *ABO*O.01.01* was the most frequent allele (*0.4944*) followed by *ABO*B.01* (*0.2555*) and *ABO*A1.01* (*0.1666).* Less frequent alleles were *ABO*A2.01* (*0.1111*), *ABO*O.01.02* alleles (*0.3888*) and *ABO*O.02.01* (*0.333*). Table 3 shows distribution of *ABO* alleles in this study and its comparison with that of other countries.

Discussion

This is the first investigation on the frequency of *ABO* alleles in a malaria-endemic city of Southern Pakistan. Twelve genotypes were identified using allele-specific primers and *ABO*O.01.01* allele

was observed as the most frequent ABO allele. High prevalence of O phenotype and *O1* allele provides an insight into a probable selective pressure exerted by malaria in this region.

Cserti C and Dzik W in 2007 described the underlying mechanism that favors better survival of O-red cells in malaria-endemic regions [4]. Accordingly, infected red cells express adhesive molecules called *Plasmodium falciparum* erythrocyte membrane protein (*Pf* EMP-1) having two domains that are Duffy binding like proteins and Cysteine-rich interdomain region-1 which bind respectively with blood group A/B determinants and CD 36 on platelets and endothelium [4]. It is now known that rosetting is an important virulence factor of *Plasmodium falciparum* causing severe malaria through cytoadhesion of parasitized red cells to other red cells, platelets and endothelium [6]. The influence of ABO type on rosetting was observed very early in 1995 by Rowe et al during their study on 154 fresh *Plasmodium falciparum* isolates from pediatric patients in Kenya and reported a low frequency of 2% rosette formation in O-red cells (range 0-45%) compared to a high of 7% (range 0-82%) in A-red cells (P-value <0.01) [19].

Numerous studies have reported dominance of blood type O in regions where malaria transmission is high. A report from India observed a higher frequency of complicated malaria in 119 of 247 patients (49%) having type B while type O was more prevalent in 174 healthy individuals and those having less severe malaria (52/106 or 49%) [20]. Similarly, blood type O was observed as the most frequent type in uncomplicated malaria (45.7%) and in healthy controls (41%) in Ethiopia while type A, B and AB were predominant ABO groups in those having cerebral malaria, severe anemia and shock [21]. Moreover, this protective effect of O was maintained even after adjusting for hemoglobin genotype (OR=0.743 (CI (95%): 0.566-0.976) as reported in 3100 children in Southwest Nigeria having sickle cell trait (14.4%), sickle cell anemia (1.6%), hemoglobin C trait (5.8%) and hemoglobin S/C disease (0.8%) [22]. In contrast to severe malaria, a recent metanalysis did not find any difference in distribution of blood groups in asymptomatic or uncomplicated malaria and reported a higher prevalence of active placental infection in primigravids having blood group O compared to A [23]. Therefore, it is likely that protective effect of blood group O is seen in patients having severe/complicated malaria but not in those who are carriers or having non-severe malaria or those women who are pregnant. Like the control group in the above studies, we observed blood group O predominantly in general population (blood donors) in a malaria endemic area.

Relationship between *ABO* genotype and falciparum malaria is largely unknown. *ABO*A1.01* allele is usually employed as the reference against which all other alleles are compared. *ABO*O.01.01* allele varies from the *ABO*A1.01* allele by a single base deletion at nucleotide 261 corresponding to amino acid 87 of the A transferase. Similarly, *ABO*B.01* allele differs from the *ABO*A1.01* allele by seven single base substitutions within the coding sequence at nucleotide 297, 526, 657, 703, 796, 803 and 930 [24]. There is extensive heterogeneity underlying *ABO* alleles that produce normal ABO blood groups and its subgroups such as Ax, A2, and B3. More than 300 *ABO* alleles or gene variants are listed, most of them arise due to single nucleotide polymorphisms driving amino acid changes, nucleotide deletions or insertions, and stop codons, whilst others are formed by recombination between two *ABO* alleles [25]. *ABO*O.01.01* allele is supposedly the most ancient one and might have been selected under pressure by malarial parasites [4].

A Brazilian report on 142 blood donors and 98 patients having uncomplicated falciparum malaria found a high frequency of *ABO*O01O01* in 134/240 individuals but did not observe a significant difference in the *ABO* allelic frequency (p-value 0.7) between general population and patients [26]. These findings were consistent with those reported in the meta-analysis of blood phenotypes among patients with asymptomatic malaria [23].

Figure 1 shows *ABO* allelic frequency in countries with no malaria and those having malaria endemicity as per world malaria report 2021[1]. This figure depicts that region with malaria endemicity has preponderance of *O1* allele and a lower frequency of blood group *A1* compared to malaria-free countries. This is further elaborated in Table 3 that summarizes the ratio of allelic frequency of O vs. non-O groups. In malaria-endemic areas, we observed ≥ 3 times higher frequency of *O* allele compared to *A* allele while in malaria-fee countries, *O: A* allelic ratio was less than three signifying higher proportion of *O* allele in countries with malaria endemicity. However, the proportions in *O* and *B* alleles between malaria-free and -endemic countries were similar. Like ABO genotype, similar finding was reported earlier for ABO phenotype by Cserti C and Dzik W in 2007 whereby a ratio of type O to A was reported as <1.0 in non-malaria-endemic areas in contrast to endemic areas where this ratio was >1.0 [4].

Strengths and limitations:

The study determines the ABO genotype in Pakistani population for the first time and demonstrated the high frequency of *O* allele in a malaria endemic area. This small-scale study may be the data base for further research work in regions with variable degree of malaria transmission. We plan further research work to correlate the severity of malaria with blood types in different subset of vulnerable population like children under 5 years of age, pregnant women, anemic and malnourished individuals.

Conclusion

In conclusion, the present study demonstrated a high prevalence of *O* allele in a malaria-endemic area in Pakistan. Allelic frequency of *ABO* will help in establishing a database for future studies in various cities with variable degree of malaria transmission.

Abbreviations

AKUH: Aga Khan University Hospital;

G6PD: glucose-6-phosphate dehydrogenase;

ICT: immuno-chromatographic test;

ISBT: International Society of Blood Transfusion;

SSP-PCR: Sequence-Specific primers- Polymerase chain reaction;

WHO: World Health Organization.

Declarations

Acknowledgment:

Authors are grateful to the participants, research associates (Ms. Shahnila Alidina and Ms. Amna Nasir) and to the department of Pathology and Laboratory Medicine, Aga Khan University for technical and financial supports.

Authors' contributions Study design: BM; Collection and assembly of data: SR; Data analysis and interpretation: SR; Draft manuscript: SR and BM; Manuscript review and revisions: BM; Final approval of manuscript: SR and BM. All authors read and approved the final manuscript.

Funding: This study was supported by the department of Pathology and Laboratory Medicine, The Aga Khan University, Pakistan.

Availability of data and materials:

Not applicable

Code availability:

Not applicable

Declarations:

Ethical Compliance:

The research was approved for study by the institutional ethical review committee of Aga Khan University (Review reference number # 2020-4810-10803).

Consent to participate:

Written approval was taken from all participants for testing their samples.

Consent for publication:

Not applicable

Competing interests:

The authors declare that they have no conflict of interest relevant to this article.

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Tables

Table 1 ABO phenotype and genotype relationship among 90 individuals

Phenotype	N (%)	Genotype	N (%)
A1	21 (23.3)	*O.01.01/A1.01*	13
A1		*O.02.01/A1.01*	1
A1		*A1.01/ A1.01*	3
A1		*O.01.02/A1.01*	2
A2/ A Sub		*O.01.01/A2.01*	2
B	31(34.4)	*O.01.01/B.01*	22
B		*B.01/ B.01*	7
B		*O.01.02/B.01*	2
O	30 (33.3)	*O.01.01/ O.01.01*	22
O		*O.01.01/ O.02.01*	5
O		*O.01.01/ O.01.02*	3
AB	8 (10)	*A1.01/B.01*	8
All	90 (100)	All	90(100)

Table 2. Frequency distribution of 180 ABO alleles in the study population

Alleles	n	Frequency
ABO*A1.01	30	*0.1666*
ABO*A2.01	2	*0.0111*
ABO*B.01	46	*0.2555*
ABO*O.01.01	89	*0.4944*
ABO*O.01.02	7	*0.0388*
ABO*O.02.01	6	*0.0333*

Table 3. Comparison of O and non-O alleles in different countries with prevalence of malaria

Malaria distribution	Location	n	*O-allele frequency*	*Non-O allele frequency*		Reference	Allelic ratio	
				A-	*B-*		*O/A*	*O/B*
	Cameroon	14546	*0.6978*	*0.1605*	*0.1416*	[27]	4.3	4.9
	Ethiopia	6471	*0.6859*	*0.1714*	*0.1433*	[28]	4.0	4.7
	India	100	*0.5500*	*0.1850*	*0.2650*	[29]	3.0	2.1
	Pakistan	90	*0.5665*	*0.1777*	*0.2555*	-	3.2	2.2
	Saudi Arabia	107	*0.6308*	*0.2103*	*0.1589*	[30]	3.0	4.0
Malaria-free	Australia	294	*0.6581*	*0.2432*	*0.0986*	[31]	2.7	6.7
	China	417	*0.5599*	*0.2133*	*0.2062*	[32]	2.6	2.7
	Egypt	100	*0.5000*	*0.2917*	*0.2083*	[33]	1.7	2.4
	Europe	98	*0.5817*	*0.2092*	*0.1276*	[34]	2.8	4.6
	Russia	442	*0.5500*	*0.2550*	*0.2000*	[35]	2.1	2.7

Figures

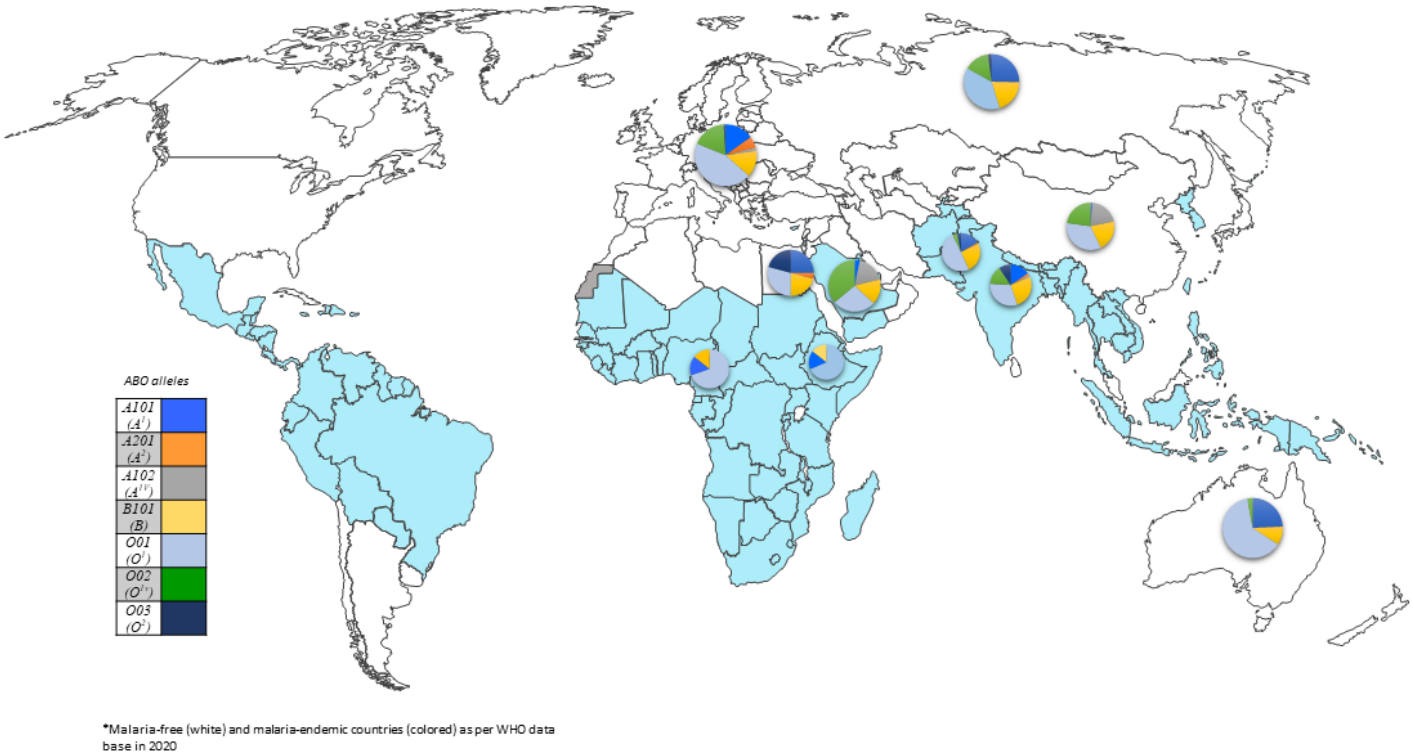

Figure 1

World map showing distribution of malaria in various countries of the world. Those that report zero indigenous cases of malaria for at least 3 consecutive years (such as Egypt, Oman, Turkey etc.) are considered to have eliminated and others that report no infection since 2000 are all shown as malaria-free countries. *ABO* allelic frequency is shown as a pie chart for the respective country

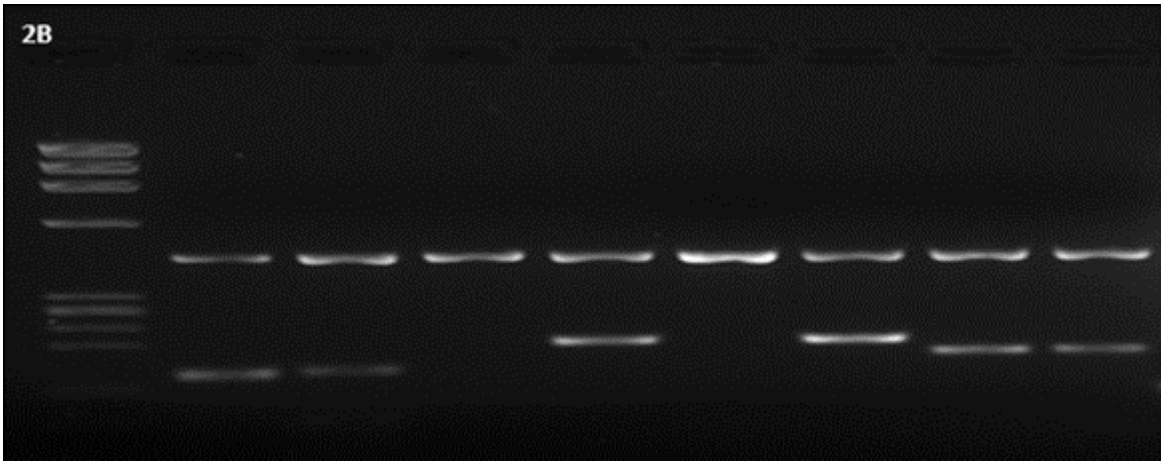

Figure 2

Results of Sequence-Specific Primers (SSP)-PCR by ABO-type kit: Figure 2 A-E showed identification of *O.01.01/A1.01, O.01.01/A2.01, O.01.01/B.01, O.01.01/O.01.01, A1.01/B.01* respectively.

Lane L: 100 bp marker, lane 1: *O.01.01* allele (134bp), lane 2: non-*O.01.01* allele (140bp), lane 3: *O.02.01* allele (194bp), lane 4: non-*O.02.01* allele (193bp), lane 5: *B.01* allele (195bp), lane 6: non-*B.01* allele (194bp), lane 7: *A.2.01* allele (172bp), lane 8: non-*A.2.01* allele (173bp).