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RARE SUBGROUP OF BLOOD GROUP A IN FIRST-TIME BLOOD DONOR

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Abstract

Introduction: A subgroup is a phenotype with variations in the structure or in the number of antigens on the red blood cell membrane. Blood group A has 20 subgroups, of which A_1 and A_2 are the most common. Other subgroups of blood group A are: A_3, A_x, A_{end}, A_m, A_y and A_{el}.

Aim: To show the importance of A_{el} subgroup because it is rare and causes ABO discrepancy. To emphasize the importance of using two techniques, serological and molecular, to confirm it.

Materials and methods: A blood sample was collected from the donor in a tube containing anticoagulant. In microgel cards, 50 µl of examined red blood cells were pipetted for forward typing, and 50 µl of examined plasma together with A_1 and B red blood cells were pipetted for reverse typing. These microgel cards were incubated at 4°C and 37°C. Adsorption-elution test was performed. DNA was extracted from peripheral blood with Promega kit. ABO genotyping was performed using the RT PCR BAG ABO ERY Q kit. The interpretation of the results was done with PlexTyper.

Results: The molecular analysis detected *ABO*A1.01/O1.01.01* genotype that indicates A_1 blood group. The A antigen was confirmed with the adsorption-elution test. Absence of agglutination with anti-A and anti-B reagents and presence of an irregular anti-A_1 antibody was detected with serological tests. The serological tests refer to A_{el} subgroup.

Conclusion: Subgroups of the ABO blood group system are rare but significant because they lead to discrepancies. To resolve these discrepancies, serological and molecular techniques are used.

Keywords: ABO blood group system, subgroup, A_{el}, ABO genotyping, *AEL.02*

Introduction

The ABO system is the most important of all blood groups in both transfusion and transplant medicine. Antigens of this blood group system are oligosaccharides. In addition to the membrane of the red blood cells, these antigens are present in soluble form in the body fluids and in many different tissues and organs[1]. The ABO blood group system consists of four antigens (A, A_1, B, AB) and six basic phenotypes (A_1, A_2, B, O, A_1B, A_2B). There are many variations in the ABO phenotypes as a result of quantitative and qualitative modifications of the A and B antigens that are genetically determined. The term "subgroup" refers to

phenotypes with variations in the structure or in the number of antigens on the red blood cell membrane. Subgroups of blood group B are very rare, whereas blood group A has 20 subgroups, of which A_1 and A_2 are the most common (99%)[2]. A subgroups are more frequent in White populations, while B subgroups are more frequent among the Japanese population[3]. Other subgroups of blood group A are: A_3, A_x, A_{end}, A_m, A_y and A_{el}, and they all account for about 1%. These subgroups are characterized by weak expression of the A antigen, reduced activity of the enzyme N-acetyl-D-galactosamine, reduced branching of the oligosaccharide chains and presence of an irregular anti-A_1 antibody in certain subgroups. The characteristics of individual A subgroups are as follows:

The frequency of the A_3 subgroup is about 1 in 1,000. A_3 red blood cells characteristically demonstrate a mixed-field pattern of agglutination with anti-A and anti-A,B reagents. Mixed-field agglutination can be defined as small agglutinates within predominantly unagglutinated red blood cells. The estimated number of A antigen sites is approximately 35,000 per red blood cell. An anti-A_1 antibody may be present in the serum of A_3 individuals, and A substance is detected in the saliva of A_3 secretors[4,5].

The A_x subgroup is less frequent than A_3, with frequency of about 1 in 40,000. A_x red blood cells characteristically are not agglutinated by anti-A reagent but do agglutinate with the most anti-A,B reagents. The estimated number of A antigen sites is approximately 4,000 per red blood cell. A_x individuals usually produce an anti-A_1 antibody in their serum. Routine secretor studies detect the presence of only H-substance in A_x secretors[5-8].

A_{end} red blood cells characteristically demonstrate very weak mixed-field agglutination with anti-A and anti-A,B reagents. The estimated number of A antigen sites on the few agglutinable red blood cells is approximately 3,500 per red blood cell. Secretor studies detect the presence of only H substance in the saliva of A_{end} secretors. An anti-A_1 antibody is found in some A_{end} sera[5,7,9].

A_m is a very rare subgroup. A_m red blood cells are not typically agglutinated by anti-A and anti-A,B reagents. The estimated number of A antigen sites varies from 200 to 1,900 per red blood cell in A_m individuals. These individuals usually do not produce an anti-A_1 antibody in their sera. Normal quantities of A and H substance are found in the saliva of A_m secretors[5,10].

A_y red blood cells are not agglutinated by anti-A and anti-A,B reagents. Saliva secretor studies demonstrate H and A substance, with A substance present in below-normal quantities. A_y individuals usually do not produce an anti-A_1 antibody[5,11].

A_{el} is a rare blood type that expresses the smallest amount of A antigens among the subgroups. The frequency of the A_{el} phenotype has been estimated to be about 0.001% in the Korean population and 0.0049% in the Japanese population. *AEL.02* is the most frequently identified allele. Red blood cells of the A_{el} subtype show no agglutination at all with the anti-A and anti-A,B reagents. The presence of A antigen on the A_{el} red blood cells is demonstrable only by the adsorption-elution test which is known as the most sensitive method for detecting the A subgroups. A_{el} individuals usually produce an anti-A_1 antibody. Secretor studies demonstrate the presence of only H substance in the saliva of A_{el} secretors[12-16].

Table 1. Serological characteristics of subgroups in blood group A

Subgroup of A	Red blood cell reactions with : Anti - A	Anti – A,B	Anti – A1	Anti - H	ABO substance in saliva	Anti – A_1 in serum
A_3	2+/ + mf	2+/ + mf	0	4+	A, H	No
A_x	0/+	2+/+	0	4+	H	Often
A_{el}	0	0	0	4+	H	Sometimes
A_{end}	+	+	0	4+	H	Sometimes
A_{finn}	+	+	0	4+	H	Yes
A_{bantu}	+/ (+)	+/ (+)	0	4+	H	Yes
A_m	0/+	0/+	0	4+	A, H	No

A_Y	0	0	0	4+	A, H	No

The ABO subgroups lead to blood group discrepancies that cause difficulties in the ABO typing. The most sensitive method for detecting the A antigen on the red blood cells is the adsorption- elution test.[4,9] Serological tests complement molecular analyses[17,18]. Isolation of DNA from peripheral blood and application of PCR techniques have enabled ABO genotyping.[19,20] The ABO genotyping identifies an individual's specific ABO blood group alleles at DNA level. Genotyping allows precise identification of subgroups and resolution of discrepancies. It is generally more costly and often is used as a supplementary tool rather than as a first-line test[21-23]. When the genotype is inconsistent with the phenotype, the next step is DNA sequencing. DNA sequencing determines the precise order of nucleotides comprising adenine, cytosine, guanine and thymine in a DNA segment or even in the entire genome. Sequencing is superior in detecting rare and novel mutations[24]. Table 2 illustrates the mutations that lead to A_{el} phenotype. It is known that eight *AEL* alleles exist - *ABO*AEL.01-08*. All of these arise as a result of mutations in exon 7 of the ABO gene, with the exception of *ABO*AEL.04* (Table 2-https://www.isbtweb.org/resource/001aboalleles.html) The *AEL.01* and *AEL.02* alleles are present in the Korean population, and the *AEL.03* allele was identified by molecular genetic analysis in 2014 for the first time in the same population[12,13,15]. *AEL.02* is the most frequently identified allele in the A_{el} subgroup[12,13,15,15].

Table 2. Molecular bases associated with A_{el} phenotypes

Phenotype	Allele name	Nucleotide change	Exon	Predicted amino acid change
Ael	ABO*AEL.01	c.804dupG	7	p.Phe269Valfs*124
Ael	ABO*AEL.02	c.467C>T; c.646T>A; c.681G>A	7	p.Pro156Leu; p.Phe216Ile
Ael	ABO*AEL.03	c.804delG	7	p.Phe269Serfs*20
Ael	ABO*AEL.04	c.374+5G>A	Intron 6	Altered splicing
Ael	ABO*AEL.05	c.467C>T; c.767T>C	7	p.Pro156Leu; p.Ile256Thr
Ael	ABO*AEL.06	c.425T>C; c.467C>T	7	p.Met142Thr; p.Pro156Leu
Ael	ABO*AEL.07	c.467C>T; 681G>A; 771C>T; c.829G>A	7	p.Pro156Leu; p.Val277Met
Ael	ABO*AEL.08	c.467C>T; c.804dupG	7	p.Pro156Leu; p.Phe269Valfs*124

Material and methods

The Department of Immunohematological Testing of Blood Donors at the Institute for Transfusion Medicine - Skopje received a blood sample in a tube containing anticoagulant for blood group typing of a first-time blood donor aged 18 years. The ABO blood group typing was done using the Bio Rad IH 1 000 analyzer, Bio Rad micro gel cards and Bio Rad reagents. The blood sample was centrifuged at 3 500 rpm for 1 minute. In microgel cards for forward blood group typing using the column agglutination technique (CAT), 50 µl 0.8% suspension of examined red blood cells were pipetted in the columns with incorporated anti-A, anti-B and anti-D VI+ reagent. Afterwards, microgel cards were centrifuged at 1000 rpm for 10 minutes. In micro gel cards for reverse blood group typing using the column agglutination technique, 50 µl 0.8% suspension of examined red blood cells were pipetted in the columns with incorporated anti-A, anti-B and anti-D reagents, and 50 µl of examined plasma and 50 µl 0.8% suspension

of A_1 and B reagent red blood cells were pipetted for the reverse typing. Afterwards, microgel cards were centrifuged at 1000 rpm for 10 minutes.

The blood sample was sent to the Reference Immunohematology Laboratory at the Institute for Transfusion Medicine-Skopje for further investigations. The blood group typing was repeated with Grifols analyzer, Diagnostic Grifols S.A micro gel cards and SeriGrup Diana A_1/B reagents. Along with automated blood group typing, a manual technique using Bio Rad microgel cards for reverse blood group typing and tube testing was applied. In microgel cards for reverse blood group typing, 50 µl 0.8% suspension of examined red blood cells were pipetted in the columns with incorporated anti-A, anti-B and anti-D reagents, and 50 µl of examined plasma and 50 µl 0.8% suspension of A_1 and B reagent red blood cells were pipetted for the reverse typing. Afterwards, microgel cards were incubated at 37°C and 4 °C for 30 minutes and centrifuged at 1000 rpm for 10 minutes.

In addition, the sample was tested using the tube technique as the gold standard for ABO blood group typing. ABO blood typing in tubes is a manual, highly sensitive method involving forward and reverse grouping to detect antigens on the red blood cells and antibodies in the plasma. The tubes were labeled for anti-A, anti-AB, anti-B, Anti-D. One drop of the respective reagent was added to each tube. A 3-5% suspension of the donor's red blood cells in isotonic saline was prepared, and one drop of the red cell suspension was added to each tube. In two additional tubes, two drops of the donor's plasma were mixed with known A_1 and B reagent red blood cells. One pair of tubes was incubated at 4 °C for 30 minutes, and the other was left at room temperature. Afterward, the tubes were centrifuged at 1,500 rpm for one minute.

A blood sample in a tube with anticoagulant was sent to the Department for Immunogenetics, Transplantation Immunology and Molecular Biology at the Institute of Transfusion Medicine - Skopje for ABO genotyping. DNA from peripheral blood was extracted with the Promega kit. Genotyping was performed using the RT PCR BAG ABO ERY Q kit. Interpretation of the results was done with the specific software PlexTyper.

Adsorption and elution testing with heat was performed in the Reference Immunohematology Laboratory at the Institute of Transfusion Medicine - Skopje. First, the tested red blood cells were washed six times with saline. After washing, in a clean test tube, two parts of anti-A reagent were added to one part of washed red blood cells and incubated for 4 hours at 20 - 24°C. The tube was then centrifuged at 3000 rpm, and the supernatant was removed. The red blood cells were washed six times with saline. An equal amount of saline was added to the washed red blood cells. The test tube was placed in a heated water bath at 56°C for 10 minutes. Immediately afterwards, the tube was centrifuged and the eluate was separated. 50 µl reagent red blood cells of O, A_1 and B type and 25 µl of the eluate were pipetted into a neutral gel card. Then, the neutral gel card was centrifuged at 1000 rpm for 10 minutes.[1,3]

Results

In the Department for Immunohematological Testing of Blood Donors microgel cards for forward blood group typing detected absence of agglutination with anti-A and anti-B reagents and agglutination with a strength of 4+ with anti-D VI+ reagent indicating blood group O positive. In microgel cards for reverse blood group typing, no agglutination was observed with anti-A and anti-B reagent in forward typing, while agglutination with a strength of 4+ with reagent red blood cells of type B was present in reverse typing. This indicates an ABO blood group discrepancy due to a mismatch between the results of the forward typing (testing red blood cells with known anti-A and anti-B reagents) and the reverse typing (testing plasma with commercially prepared reagent red blood cells of type A_1 and B). In the tested blood sample, the forward typing corresponded to blood group O (absent reaction of the tested red blood cells

with anti-A and anti-B reagents), while the reverse typing indicated blood group A (presence of anti-B antibodies in the donor's plasma that agglutinate the red blood cells of type B).

Next, in the Reference Immunohematology Laboratory, the ABO blood group discrepancy was confirmed with both techniques, automated and manual. The blood group typing was repeated using tubes and microgel cards for reverse typing. In microgel cards for forward typing, no agglutination was detected with anti-A and anti-B reagents, while agglutination with a strength of 4+ with anti-D VI- reagent indicated blood group O positive. In reverse typing, agglutination with a strength of 4+ with reagent red blood cells of type B was present corresponding to blood group A (Figure 1). In the tubes incubated at room temperature, no agglutination was visible with anti-A, anti-AB, anti-B reagents, while agglutination was present with anti-D reagent. In reverse typing, only agglutination with reagent red blood cells of type B was visible. The forward typing corresponds to O positive blood group, while the reverse typing to blood group A. Therefore, the ABO blood group discrepancy serologically was detected with the tube and the microgel card testing.

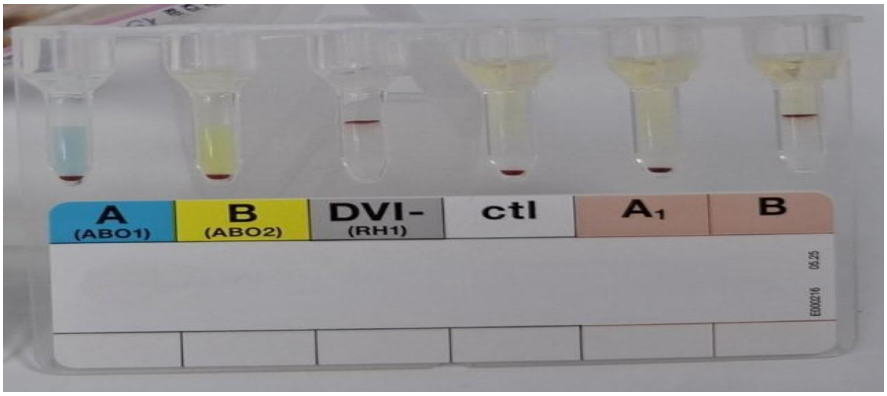

Fig. 1. Blood group typing in microgel cards for reverse typing

In microgel cards incubated at 37ºC, forward typing showed no agglutination of the tested red blood cells with anti-A and anti-B reagents, while agglutination with strength of 4+ with reagent red blood cells of type B was present in reverse typing. The forward typing indicates blood group O, whereas the reverse typing corresponds to blood group A (Figure 2).

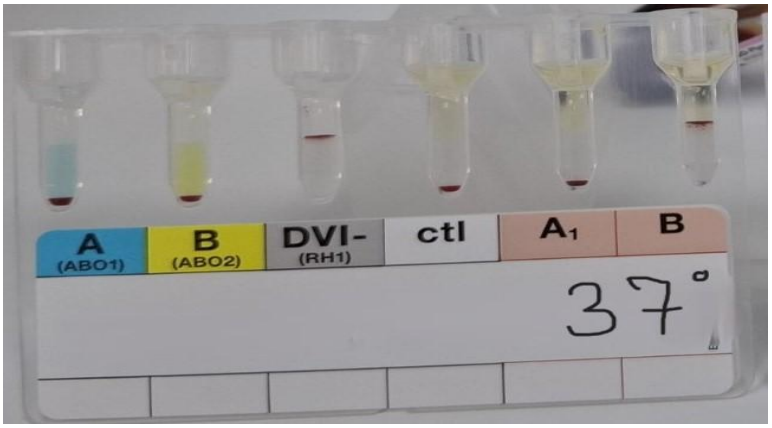

Fig. 2. Blood group typing in microgel cards for reverse blood group typing incubated at 37ºC

In microgel cards for reverse blood group typing incubated at 4ºC, forward typing showed no agglutination of the tested red blood cells with anti-A and anti-B reagents. In reverse typing, agglutination with a strength of 1+ was observed with reagent red blood cells of type

A_1, and agglutination with a strength of 4+ was observed with reagent red blood cell of type B (Figure 3). In the tubes incubated at 4ºC, no agglutination was visible with anti-A, anti-AB, anti-B reagents, while agglutination was present with the anti-D reagent. In reverse typing, weak agglutination with reagent red blood cells of type A_1 and strong agglutination with reagent red blood cells of type B were observed. The forward and reverse typing corresponded to O positive blood group.

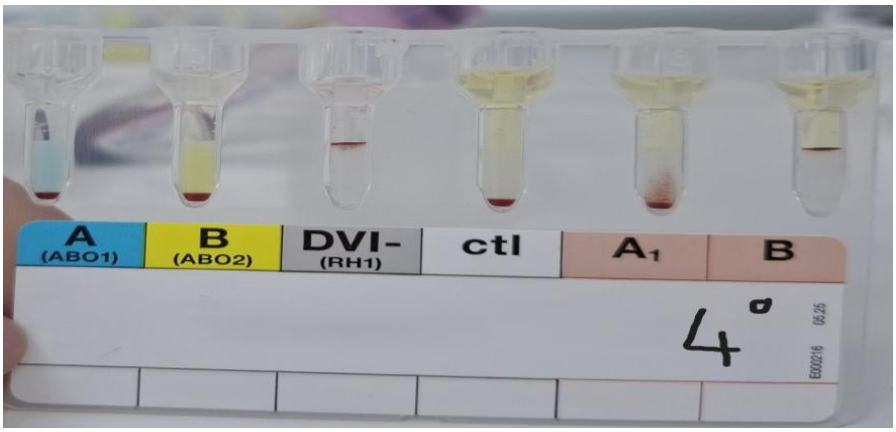

Fig. 3. Blood group typing in microgel cards for reverse blood group typing incubated at 4ºC

The adsorption and elution technique with heat confirmed the presence of A antigen on red blood cells of the blood donor (Figure 4).

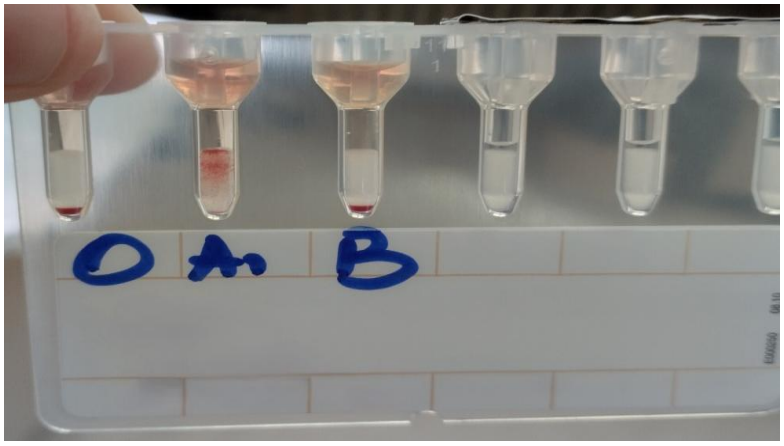

Fig. 4. Detection of A antigen with adsorption-elution test

The ABO genotyping resulted in ABO**A1.01/O1.01.01* genotype indicating an A_1 blood group. The adsorption-elution test with heat demonstrated the presence of A antigen on tested red blood cells of the donor. Serological tests detected absence of agglutination of tested red blood cells with anti-A and anti-B reagents and presence of an irregular, cold anti-A_1 antibody in the serum corresponding to the A_{el} subgroup of blood group A.

Discussion

A_{el} is a rare subgroup of the blood type A that expresses the smallest amount of A antigens[12,13,16]. Red blood cells of the A_{el} subtype show no agglutination with anti-A and anti-A,B reagents. A antigens on the red blood cells can only be detected by the adsorption-elution test, which is known as the most sensitive method for detecting A subgroups[12,13]. Typical for the A_{el} subgroup is the presence of an irregular anti-A_1 antibody in the serum, which is usually

less reactive with the corresponding reagent red blood cells than the natural anti-B antibody[4,12-14]. This was also confirmed in our case. Specifically, agglutination with a strength of 1+ was obtained with the reagent red blood cells of type A_1 due to the presence of a cold, irregular anti-A_1 antibody, and agglutination with a strength of 4+ was observed with the reagent red blood cell of type B due to the presence of a natural anti-B antibody after incubation of microgel cards for reverse blood group typing at 4 ºC. To resolve this ABO blood group discrepancy, several serological tests were performed, as mentioned above, of which the adsorption-elution testing with heat was the key and decisive one. The adsorption and elution technique with heat proved the presence of A antigen on the red blood cells of the blood donor. In the presented case, the *ABO*A1.01/O1.01.01* genotype was obtained, which indicates blood group A_1. The genotype was inconsistent with the phenotype. ABO sequencing is needed to resolve the molecular basis of the A_{el} subgroup in the blood donor and to detect the mutation. Mutations in the ABO gene affect the activity of the glycosyltransferases, causing less effective conversion of the H substance into A or B antigens, resulting in modified or weak expression of A or B antigens on the surface of the red blood cells. Detection of the mutation alone is not decisive for differentiation of various subgroups. The serological tests using tubes and microgel cards, adsorption-elution testing with heat, saliva testing for secretor status, usage of anti-H lectin (Ulex europaeus) and anti-A_1 lectin (Dolichos biflorus) are crucial for distinction of different ABO subgroups (Table 1). Serologically, the most sensitive method for detecting A antigen on red blood cells in different A subgroups is the adsorption-elution test[4,9]. Serological tests complement molecular analyses[17,18]. Molecular analyses are applied in polytransfused and transplanted patients and in resolving ABO discrepancies. They provide confirmation of the serological test results, improve the accuracy in determination of blood groups, and help in prevention of potentially fatal transfusion reactions. Genotyping is often used as supplementary tool rather than as a first-line test[21-23]. DNA sequencing is superior for detecting rare and novel mutations[24].

The identification of the A_{el} subgroup in a first-time blood donor using serological and molecular techniques is of particular importance because retrospectively since June 2017 this subgroup had not been detected at the Institute for Transfusion Medicine - Skopje. Since June 2017, twenty-one A_2, seven A_x, four A_{weak}, one A_3 and one A_{el} subgroups have been identified. Considering the very low incidence of the A_{el} subgroup in the Macedonian population and the implementation of molecular techniques at the Institute for Transfusion Medicine since June 2017, it is assumed that the A_{el} subgroup had not been previously missed during typing.

Conclusion

Subgroups of the ABO blood group system are rare but significant because they lead to discrepancies that cause difficulties in ABO typing. In order to accurately determine blood groups and prevent potentially fatal transfusion reactions, serological methods must be combined with molecular techniques, including sequencing if needed. These methods complement each other in resolving blood group discrepancies.

Conflict of interest statement. None declared.

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