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ORIGINAL ARTICLE

Establishing Blood Group Genotyping to Resolve ABO Discrepancies in Iran

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Abstract ABO discrepancies are recognized when the reactions obtained in the forward type do not "match" the reactions obtained in the reverse type. Discrepant results are often caused by a variant ABO gene. Molecular analysis is required to confirm the type of subgroups and discrepancy. In this study ABO genotyping was performed on a series of blood donors and patients to determine their definite blood groups. We examined 100 samples with ABO discrepancies from blood donors and patients referred to Tehran Blood Transfusion Center between October 2015 and August 2016. ABO genotyping was performed on all samples with allele specific PCR for differentiation of A, B and O alleles. Exon 6 and 7 of ABO gene were sequenced to confirm the results. The genotyping of donor/patients samples with discrepant results of ABO blood typing consisted of 61 cases of A_2 and A_2B, 3 cases of *B302* and 4 cases of *Aw06*. Genotyping of 6 samples that had extra antibody in their serum (AB blood group) confirmed the cell type reaction results. 6 samples that had shown a very weak reaction with anti-AB (similar to O blood group) and had no anti-A in their serum were genotyped as O_1O_2. Blood group genotyping laboratory provides an efficient service for evaluation of ABO discrepancies and resolve the problems encountered in serology reactions.

Keywords ABO discrepancy · Blood group · Genotyping

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Introduction

The ABO is the first and most important blood group system that must be correctly identified before transfusion and transplantation procedures. ISBT has reported that 36 human blood group system genes have been identified by now. Consequences of both ABO incompatible transfusions and transfusion transmitted infectious (TTIs) are grave. ABO incompatible transfusions cause immediate acute reactions whereas TTIs cause delayed Transfusion reactions. So *ABO* grouping is to be considered valid and results of cell and serum grouping should agree. ABO discrepancies are recognized when the reactions obtained in the forward type "do not match" the reactions obtained in the reverse type [1–7]. Clerical and technical errors, patient conditions such as age, chimera, and diseases that affect antigen expression or antibody production and weak subgroups of ABO antigens cause ABO discrepancies [4, 5]. *ABO* genotyping is frequently used in cases of serologic discrepancy to ensure safe transfusion [8]. Almost 300 *ABO* gene variants have been described so far most of which are characterized by one or more SNPs leading to amino acid changes, stop codons and nucleotide insertions or deletions [9, 10]. The *ABO* gene codes for the glycosyltransferases which are located on the human chromosome 9 consist of seven exons. Exons 6 and 7 encode the soluble part of the ABO glycosyltransferases including the catalytic domain that mediate the expression of A and B antigens [11–13]. Several *ABO* genotyping methods including sequence-specific primer(SSP)-PCR, PCR-restriction fragment length polymorphism (PCR–RFLP) or PCR-single-stranded conformational polymorphism (SSCP) have been used for determination of different *ABO* alleles [14].Moreover, accurate *ABO* genotype results are obtained by sequencing methods based on exons

6 and7 to distinguish weak ABO antigens (A_3, A_x, B_3, etc.) [8, 10]. For the first time, we have established a blood group genotyping central lab in Iranian Blood Transfusion Organization (IBTO) and in the present study, *ABO* genotyping was performed on a series of ABO discrepant samples from Iranian blood donors and patients by PCR-SSP method. Sequencing was also carried out to confirm the results.

Materials and Methods

Blood Group Serology and DNA Extraction

One hundred ABO discrepancy blood samples in EDTA anticoagulant were received in total from blood donors and patients referred to Tehran Blood Transfusion Centre with informed consent.

ABO phenotyping was carried out with the Qwalys automated system (Diagast Company, France). The tests were repeated for discrepant results by standard tube testing method with monoclonal anti-A and anti-B reagent (LORNE laboratories, UK) for cell type and confirmed by Anti-A_1 and Anti-H (LORNE laboratories, UK). A_1, A_2, B reagent cells (from IBTO pooled cells) were used for serum type.

The whole blood was centrifuged at 2000 g for 10 min and buffy coat was separated. DNA was extracted using QIAmp DNA Mini Kit (QIAGEN, Germany). DNA concentration was measured by spectrophotometer (Thermo, U.S.A).

SSP-PCR

Sequence Specific Primer-PCR (SSP-PCR) was performed using designed primers for *ABO* alleles *O_1, O_2, A_1, A_2* and *B* [15]. Human growth hormone (hgh) was used as internal control. PCR amplification was performed in a final reaction volume of 25 µl, containing 12.5 µl of 2X PCR Master Mix (TAKARA, Japan), 80–140 ng of genomic DNA and 0.4 µM of each primer in one tube. Profile was run as the following steps: 5 cycles with initial denaturation at 95 °C for 30 s, annealing at 61 °C for 150 s and then extension at 72 °C for 15 s followed by 30 cycles with initial denaturation at 95 °C for 30 s, annealing at 63 °C for 60 s, extension at 72 °C for 15 s. Undetermined samples were analyzed by a commercial kit (ABO type variant, BAGene SSP, Germany). In BAGene kit a series of 18 reactions with allele specific primers have been used for detection of *ABO* alleles. All PCR products were analyzed on 1.5% agarose gel electrophoresis.

DNA Sequencing

Genomic DNA sequencing was done on 7 weak subgroup samples. *ABO* exons 6 and 7 were amplified using primers from Goebel et al. [10].The primer pair used for exon 6 amplified a 429-bp fragment of *ABO* gene spanning the region from the intron 5 to intron 6 and primer pairs for exon 7 amplified two 528-bp/572 bp fragments of *ABO* gene spanning the region from the intron 6 to exon 7. PCR was performed as follows: 94 °C for 120 s, 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and the final extension at 72 °C for 120 s. Sequence data were compared to *ABO* alleles listed in the dbRBC- BGMUT NCBI database [9, 10].

Results

We analyzed 100 ABO discrepant samples including 32 donors and 68 patients referred to our blood group genotype center laboratory.

Serology

The samples were divided into different categories based on the serologic reactions.

61 samples were identified as A_2 and A_2B whenever the agglutination of samples with anti-A was 4 + or weaker (2 +) and had no reaction with anti-A_1 lectin. In 16 cases out of 31 A_2 samples and 24 cases out of 30 A_2B samples, anti-A_1 was detected in their serum using A_1-cells reagent and the degree of reaction was 1 + at room temperature.

Weak reaction (w/+) was observed in cell typing of 20 cases that were considered as subgroups of A and in seven cases of weak B subgroups. There were 6 cases which were diagnosed as AB blood group in cell type reaction, but had extra antibody in their serum type reactions. Using antibody screening and identification tests revealed that the specificity of antibodies were anti-P_1 in 3 cases, anti-I in 1 case and anti-M in 2 cases. 6 samples showed a very weak reaction in cell typing with anti-AB and there was no anti-A in their serum. The serological reactions of ABO phenotyping is summarized in Table 1.

SSP-PCR

Based on reactions with allele-specific (*O_1, O_2, B, A_2*) primers reported by Gassner [15], genotyping of 61 A_2 and A_2B phenotype samples showed that 32 of them were heterozygote as *A_2O_1* and 29 as *A_2B* (Fig. 1a, b). Results of PCR-SSP were confirmed by commercial BAGene kit.

Alleles *A* and *B* were detected among 20 A subgroup and 7 B subgroup samples respectively by SSP-PCR

Table 1 Serological reactions of ABO phenotyping

ABO phenotype	Anti-A	Anti-B	Anti-AB	Anti-A_1	Anti-H	A_1 cells	B cells	O cells	No. of cases
A_2	++	0	+++	0	+++	+/0*	++++	0	18
A_2	++++	0	+++	0	+++	+	++++	0	13
A_2B	++++	++++	++++	0	++	+	0	0	21
A_2B	++	++++	++++	0	+	+/0*	0	0	9
Subgroup of B	0	++/mf	++/mf	0	+++	++++	0	0	7
Subgroup of A	+	0	++	0	++++	w/+	+++	0	20
AB with Ab	++++	++++	++++	0	0	+/0	+/0	++	6
A_{weak} without Anti-A_1	0	0	w	0	+++	0	4+	0	6

mf mixed field, *w* weak, *Ab* Antibody

*Anti A_1 was present in some cases

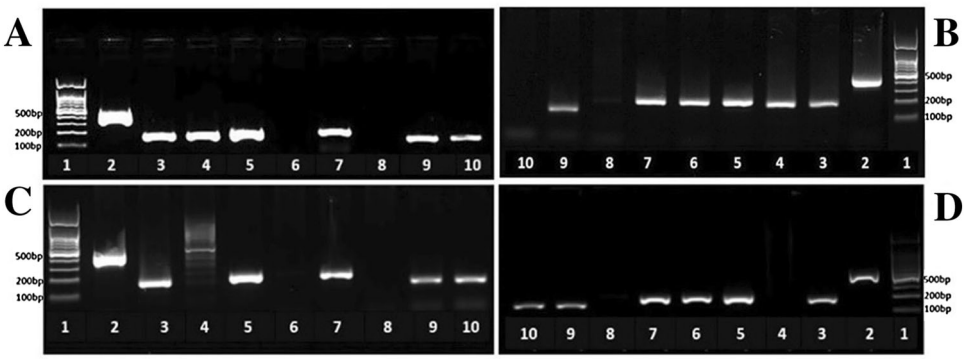

Fig. 1 Gel electrophoresis of PCR fragments amplified with allele specific primers (O_1, O_2, B, A_2). Eight PCR-reactions were used for *ABO* PCR-SSP (According to reactions represented by Gassner et al. in Table 3) [15] . O_1, O_2, B and A_2 primers were allele-specific and nonO_1, nonO_2, non$B$ and nonA_2 were non-allele specific. Allele O_1 is distinguished from non O_1 at nt. 261, O_2 from non O_2 at nt. 802, B from non B at nt. 803 and A_2 from non A_2 at nt. 1059. **1:**100 bp ladder (YT8503, Yekta Tajhiz Azma, Iran) **2:** Hgh (Human growth hormone) as an internal positive control (434 bp), **3:** nonA_2 allele(170 bp), **4:** A_2 allele(169 bp), **5:** non B allele(194 bp), **6:** B allele(195 bp), **7:** nonO_2 allele(193 bp), **8:** O_2 allele(194 bp), **9:** nonO_1 allele(137 bp), **10:** O_1 allele(139 bp). **a** in the presence of A_2 and O_1 and absent of B and O_2 the A_2O_1 pattern was confirmed. **b** In the presence of A_2 and B alleles and absent of O_1 and O_2 the A_2B pattern was confirmed. **c** In the presence of O_1 and nonO_1 alleles and absence of O_2,B and A_2 alleles the A_1O_1 pattern was confirmed. **d** In the presence of B and O_1 alleles and absence of O_2 and A_2 alleles the BO_1 pattern is confirmed

method combined with O_1 allele in heterozygote (A_1O_1, BO_1) forms (Fig. 1c, d). Genotyping of 6 samples that had extra antibody in their serum (AB blood group) confirmed the cell type reaction results. This is correlated with the specificity of additional antibodies that were shown to be due to other blood groups rather than ABO blood group system.

6 samples that had shown a very weak reaction with anti-AB (similar to O blood group) and had no anti-A in their serum, were genotyped as O_1O_2 (Fig. 2). O_2 allele was detected by specific primer for nucleotide substitution G to A at position 802. A summary of genotyping results is shown in Table 2.

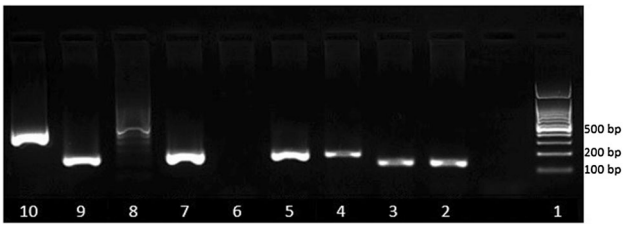

Fig. 2 PCR products amplified from O_1O_2 sample with allele specific primers (O_1,O_2,B,A_2). Allele O_1 is distinguished from nonO_1 at nt. 261, O_2 from nonO_2 at nt. 802.. **1:** 100 bp ladder (YT8503, Yekta Tajhiz Azma, Iran) **2:** O_1 allele(139 bp), **3:** nonO_1 allele(137 bp), **4:** O_2 allele(194 bp), **5:** nonO_2 allele(193 bp), **6:** B allele(195 bp), **7:** non B allele(194 bp), **8:** A_2 allele(169 bp), **9:** nonA_2 allele(170 bp), **10:** Hgh (Human growth hormone) as an internal positive control (434 bp). Allele A_2 and B band is absent so O_1O_2 pattern is confirmed

Table 2 PCR-SSP Results for ABO discrepant results

O_1 allele	Non O_1 allele	O_2 allele	Non O_2 allele	B allele	Non B allele	A_2 allele	Non A_2 allele	Genotype	Phenotype	No. of cases
+	+	−	+	−	+	+	+	A_2O_1	A_2	31
−	+	−	+	+	+	+	+	A_2B	A_2B	30
+	+	−	+	−	+	−	+	A_1O_1*	Sub group of A	20
+	+	−	+	+	+	−	+	BO_1**	Sub group of B	7
−	+	−	+	+	+	−	+	A_1B	AB with Ab	6
+	+	+	+	−	+	−	+	O_1O_2	A_{weak} without anti-A_1	6

*Not confirmed as A subgroup by PCR-SSP

**Not confirmed as B subgroup by PCR-SSP

Sequencing

By sequencing of exons 6 and 7 we analyzed 27 samples that were detected in serology as A and B subgroup and also in the SSP- PCR common *A*, *B* and *O* alleles were confirmed but their type was not specified. Among these samples four cases were found to be *ABO*Aw06* and three cases *ABO*B302* (Figs. 3 and 4a–d).

Discussion

Nowadays, due to the identification of different alleles in blood groups, using genotyping methods in various cases, such as multi-transfused patients, ABO discrepancies and different variants of the Rh blood group system, is taken into consideration. Therefore, blood bank services have also launched a molecular laboratory along with a serological one. For the first time in the Iranian Blood Transfusion Organization (IBTO), we have set up a central laboratory for blood group genotyping to help us determine the blood groups accurately. So far, little studies have been done on the genotype of blood groups in Iran [16]. We have tried to develop molecular methods in determining blood groups in this laboratory. As the first study in this laboratory, we evaluated the ABO discrepancies by molecular methods in patients and donors referred to the serology department. In clinical transfusion and transplantation medicine, ABO blood group is the most important system to ensure safe transfusion. Molecular analyzing is increasingly useful in clarifying serologic ABO discrepancies due to suspected variant phenotypes such as weak subgroups [8, 11–14].

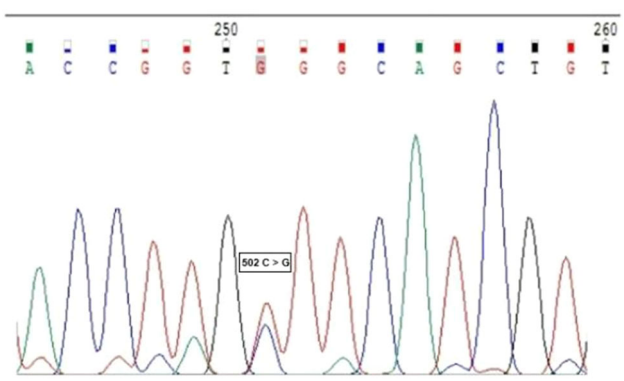

Fig. 3 Sequence of exon 7. Substitution of C with G in 502 position indicates a *Aw06* allele

In this study, most of the ABO discrepancies had weaker reaction than normal (4 +) in cell typing or there was an excess anti-A_1 in their serum. Also none of the samples reacted to the anti-A_1 lectin in this group that means the antigen A is weakened on the surface of the red blood cells. We considered these samples as A_2 that is the most prevalence A subgroup among different population [4, 5, 17]. We detected the A_2 allele in these samples by genotyping assessment. The primer was specific for the nucleotide C deletion in position 1059 [11, 12, 15, 18, 19].

As most of A_2 cases have a 4 + reaction in cell type with anti-A, finding a weak reaction in cell typing for some specimens that had classical A_2 genotype (C deletion in position 1059) can be due to additional mutations in other parts of the gene which cause the weaker expression of antigen in red blood cell surface [18–21].

In samples that had a weak reaction with anti-B in the cell type and identified in the serology as subgroup B, presence of *B* allele was confirmed by SSP-PCR reaction using a specific primer of the *B* allele (B_1O_1). However, given that these primers cannot detect *sub- B* alleles, which lead to weak expression of the antigen B at cells surface, for further examination, sequencing of exons 6 and 7 was performed. In 3 samples 646 T > A was observed. This change has been reported in the *B302* allele, which results in substitution of phenylalanine with iso-leucine (F216I) that has a drastic effect on B transferase [22–25].

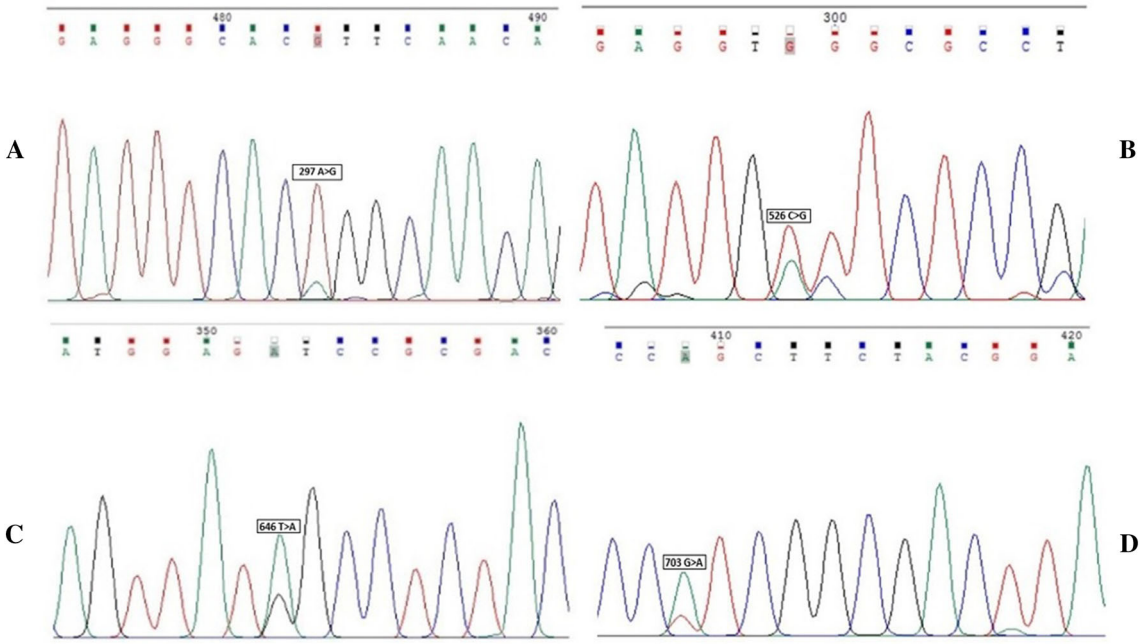

Fig. 4 Sequence of exon 6 and 7. **a** Substitution of A with G in 297 position of exon6. **b** Substitution of C with G in 526 position of exon 7. **c** Substitution of T with A in 646 position of exon 7. **d** Substitution of G with A in 703 position of exon 7. All these nucleotide changes indicate a *B302* allele

Using genotype by specific primer for the *A* allele, in the samples with a weak reaction in cell type with anti-A and presence of anti-A_1 in serum in some of them, showed that there was an *A* allele in these individuals. Given that the cell type is weaker than the reactions seen in A_2, as expected, the classic A_2 allele was not identified in this group [4, 5]. Exons 6 and 7 sequencing of these samples revealed the presence of *Aw06* allele in these cases. Changing the nucleotide 502 C > G results in substitution of arginine with glycine and thus R168G change reduce the activity of A transferase caused weak formation of antigen A on the surface of blood cells. This allele is also reported in the population of Germany and some people from Bosnia, India, Italy and Turkey [13, 22–24].

In cases where blood type AB was definitely identified in the cell type, but additional antibodies were observed in serum type, the serologic results (AB) were confirmed by genotype using SSP-PCR primers. As expected, the presence of additional antibodies in the serum type was against other antigens rather than ABO, which was determined by Ab identification methods so there was no need for further genetic and molecular studies [4].

In a number of samples that had a very weak expression of antigen A in cell typing, or was detected as O blood group, but anti-A was not present in serum type reaction, genotyping revealed the O_1O_2 allels. There are interesting reports explaining that those who have the O_2 allele can produce very little antigen A on tissue cells, which results in a lack of anti-A formation in serum of these individuals [26].

Conclusion

Blood group genotyping laboratory provide an efficient service for evaluation of ABO discrepancies and resolve the problems encountered in serology reactions.

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Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Informed Consent It was obtained from all individual participants included in the study.

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