Comparison of *ABO* genotyping methods: a study of two low-resolution polymerase chain reaction assays in a clinical testing laboratory

J.A. Keller, T. Horn, S. Scholz, S. Koenig, and M.A. Keller

The ABO blood group system is the most clinically significant system in transfusion medicine. Although serologic typing for ABO antigens is routine and reliable, molecular methods can be used to predict an ABO type in the absence of a blood specimen as well as to investigate ABO typing discrepancies often caused by ABO subgroups that cause weakened antigen expression, weak or missing serum reactivity, and/or extra red blood cell reactivity. By detecting single nucleotide variants that are hallmarks of the major *ABO* alleles, low-resolution genotyping methods can be used to make allele assignments and predict phenotypes. This approach has become a dependable tool, initially to resolve typing discrepancies identified in blood banks and donor centers and, more recently, to predict the ABO group in bone marrow transplant donors and in deceased donors of solid organs. The aim of this report is to compare two different low-resolution polymerase chain reaction (PCR)-based methods: a PCR-restriction fragment length polymorphism (RFLP) implemented based on a publication and a commercially available TaqMan-based sequence-specific primer-PCR for resolution of ABO typing discrepancies. Fifty-six peripheral blood samples from 31 patients and 25 blood donors were used to isolate genomic DNA and perform genotyping. Results of 49 of the 56 samples (87.5%) were concordant between methods, three samples yielded an unexpected banding pattern on the PCR-RFLP method, and four sample results were discordant between assays. The discordances all involved group A versus A2 discrepancies. Sanger sequencing was used as a high-resolution genotyping method to resolve discrepancies between the two low-resolution methods. This study demonstrates that, in the majority of cases, a low-resolution genotyping method can resolve an ABO discrepancy. Although there is no U.S. Food and Drug Administration–approved genotyping method for ABO determination, molecular testing for investigation of discrepancies is a useful tool for blood banks and transplant programs. *Immunohematology* 2019;35: 149–153.

Key Words: ABO, PCR-RFLP, SSP-PCR, genotyping, discrepancy

The ABO blood group system was discovered in 1900; the current nomenclature was established in 1927 by Landsteiner.[1] The system comprises four main phenotype groups: A, B, AB, and O. These groups arise from the activity of one or two distinct glycosyltransferases: A glycosyltransferase and B glycosyltransferase. These enzymes catalyze the transfer of the terminal sugar residues (*N*-acetyl-galactosamine or *D*-galactose) to the H-active chain on red blood cell (RBC) membrane glycoproteins and glycolipids—producing A antigens and/or B antigens, respectively. The lack of activity of either of these enzymes results in the group O phenotype, and the activity of both glycosyltransferases results in the group AB phenotype.

The *ABO* gene contains seven exons and spans over 20 kilobases (kb) on chromosome 9 (9q34). The reference allele is denoted as *ABO*A1.01, which encodes A glycosyltransferase, which is responsible for the A antigen. The common group O phenotype is defined by a single nucleotide deletion at c.261, causing a frameshift mutation resulting in a premature stop codon of the amino acid sequence and a subsequent null phenotype of the enzyme. Genetic variation within the *ABO* gene that changes the specificity or activity of the enzyme can result in an altered ABO phenotype. Many ABO subgroups have been classified and categorized within each phenotypic group. To date, more than 200 variant alleles have been identified in the ABO blood group system.[2]

ABO discrepancies have many causes, including recent transfusion of blood products, technical error, ABO-incompatible hematopoietic stem cell transplant, age, and medical condition.[3] Samples can demonstrate a RBC or plasma discrepancy, with weak, missing, or extra reactivity. Standard serologic methods can be adjusted to resolve such discrepancies, including changing incubation temperature or time and using lectins to differentiate reactivity for A1 versus non-A1.[4]

Patient and donor samples referred to the National Molecular Laboratory for *ABO* genotyping for investigation of serologic discrepancies in 2017 and 2018 were included in this study. In some cases, little or no serologic information was provided. For this report, we compared the results obtained from two genotyping methods when used to test samples submitted for investigation of typing discrepancies. Testing

was performed under a research protocol approved by the American Red Cross institutional review board.

Materials and Methods

Mononuclear cells from EDTA peripheral blood were used as a source of genomic DNA (QIAamp DSP; QIAGEN, Valencia, CA). Genomic DNA was polymerase chain reaction (PCR)-amplified and analyzed using two research-use-only genotyping methods: TaqMan-based sequence-specific primer (SSP)-PCR and PCR-restriction fragment length polymorphism (RFLP). SSP-PCR using RBC-FluoGene ABO Basic test kit was performed as per manufacturer's instructions (inno-train Diagnostik, Kronberg, Germany). The RBC-FluoGene ABO Basic test kit interrogates *ABO* c.261G/delG, c.802G/A, c.803G/C, and c.1061C/delC markers. Endpoint fluorescence detection was accomplished using the FluoVista analyzer and FluoGene software (v.1.5.4.0) (inno-train Diagnostik), which uses the pattern of detection of single nucleotide variants (SNVs) associated with *ABO*O.01, ABO*O.02, ABO*B,* and *ABO*A2.* The test does not assign *ABO*A1.* When SNVs associated with *A2, O1, O2,* and *B* alleles are not detected, the software predicts presence of an A allele with the stated limitation that the most likely result is A1 but that rare alleles cannot be ruled out.

PCR-RFLP interrogating of nucleotides c.261G/delG, c.467C/T, c.703G/A, and c.1096G/A, designed to discriminate between the common alleles *A1, A2, B, O1,* and *O2,* was performed as described by Olsson and Chester.[5] In a subset of cases, Sanger sequencing of *ABO* exons 1–7 was performed (Grifols Immunohematology Center, Grifols Diagnostic Solutions, San Marcos, TX). In one case, PCR products of *ABO* gene fragments were cloned into plasmid vectors and subjected to Sanger sequencing to determine the phase when multiple SNVs were detected and to facilitate allele assignments. Sanger sequencing was performed on one sample by the RBC-FluoGene kit manufacturer (inno-train Diagnostik) using Big Dye Terminators 3.1 and the ABI3130 Sequencer.

Results

TaqMan-based SSP-PCR and PCR-RFLP were performed for each sample and genotype-predicted phenotypes were compared in the context of the serologic information provided. The results are summarized and described in Table 1 for the patient and donor cohorts. Because FluoGene and RFLP methods are both low resolution, there are several limitations

Table 1. Summary of concordance between SSP-PCR and PCR-RFLP

Prediction by SSP-PCR	Total subjects, N	Patients, N	Donors, N	Concordant with PCR-RFLP N (%)
A/A	1	1	0	1 (100)
A/B	2	2	0	2 (100)
A/O1	28	15	13	22 of 28 (78.5)
A/O2	1	1	0	1 (100)
A2/B	6	4	2	6 (100)
A2/O1	7	5	2	6 of 7 (86)
B/B	1	0	1	1 (100)
B/O1	6	2	4	6 (100)
B/O2	1	1	0	1 (100)
O1/O2	3	0	3	3 (100)

SSP = sequence-specific primer; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

to each, including inability to rule out subgroups. The presence of a subgroup could cause a different genotype result. One difference between the two methods is that TaqMan-based SSP-PCR calls all non-*A2,* non-*B,* non-*O1,* and non-*O2* alleles as group A whereas the RFLP interrogates the SNV at c.467, which can aid in discriminating between groups A2 and A1. Both PCR methods can potentially fail to generate a genotype assignment when an unexpected pattern of amplification is observed. The samples that were discordant or gave unusual banding patterns on RFLP were resolved by high-resolution *ABO* sequencing to determine a definitive genotype (Table 2).

Of the 56 samples genotyped by PCR-RFLP and TaqMan-based SSP-PCR, 49 (87.5%) gave concordant results. The PCR-RFLP yielded an unusual banding pattern in three (5.4%) of the samples (cases 1–3); this result is often associated with *ABO* variant alleles, which were found in all three samples by high-resolution testing with the predicted phenotype changing in only one of these samples (case 1). In this sample, the SSP-PCR *ABO*A* result is due to a positive non-*O1* reaction but cannot be explained by the variant allele detected by sequencing. This sample was further characterized by Sanger sequencing which confirmed the sequencing results of *ABO*O1,* but no additional variants were identified that might explain the SSP-PCR result. Considering this unusual and unexpected finding, one could speculate that either *ABO* chimerism or a novel hybrid allele is present in this patient.

Four samples (7%) (cases 4–7) gave discordant results when comparing FluoGene with RFLP; all involved *A* versus *A2* alleles. One donor sample (case 7) was predicted to be group

Table 2. Summary of result for samples with discordant types between SSP-PCR and PCR-RFLP, grouped by SSP-PCR result

Case	Result by TaqMan-based SSP-PCR	Subject	Ethnicity	Result by PCR-RFLP	Sanger	Serology
1	A/O1	P28	Not provided	UBP	ABO*O.01.01/ABO*O.01.09	None
2	A/O1	P31	African American	UBP	ABO*A1.02/ABO*O.01.09	None
3	A/O1	P32	White	UBP	ABO*A1.01/ABO*O.01.26	Rouleaux demonstrating. Saline replacement removes reverse type reactivity.
4	A/O1	P30	African American	A2/O1	ABO*O.01/ ABO*IVS1+5861G,467T	Front type: group O Back type: group A
5	A/O1	D23	Hispanic	A2/O1	ABO*O.01.09/ ABO*IVS1+5860G	Back type: A1 and A2 RBCs (0−w+)
6	A/O1	D25	African American	A2/O1	ABO*O.01/ ABO*IVS1+5861G,467T	Front type: group O, anti-A,B reactive Back type: group A
7	A2/O1	D24	White	A1/O1	ABO*AW.02/ABO*O.01.01	Front type: group O, anti-A,B reactive Back type: group O with weakly reactive A2 RBCs (1+). Note: O RBCs reactive at room temperature incubation

SSP = sequence-specific primer; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; P = patient; UBP= unusual banding pattern; D = donor; RBCs = red blood cells.

A2 by FluoGene and A1 by RFLP; variant testing identified a rare *ABO*AW.02* allele that likely caused the discordant result. The other three samples (one patient, two donors) carried an *A2* allele by PCR-RFLP and an *A* allele by SSP-PCR. Again, high-resolution testing identified variants that likely resulted in the discordance between the two methods. This finding demonstrates that the presence of c.1061delC may be a more reliable marker to detect an *A2* allele and c.467C>T is prone to a false-positive A2 call in a variant allele containing this SNV. Both methods have limitations with regard to a correct genotype call in the presence of an *ABO* variant allele.

Four additional patient samples (not listed in Table 2) had high-resolution testing as per customer request because of a suspected subgroup from serologic typing; three of them were found to contain variant alleles. All samples were concordant between FluoGene and RFLP. One sample was found to carry variant *O* alleles, changing the predicted phenotype of group A from the initial low-resolution methods.

Discussion

We report the concordance of two low-resolution methods for *ABO* genotyping, comparing the predicted phenotype from an established PCR-RFLP method with that from a commercial TaqMan-based SSP-PCR kit when used for testing genomic DNA samples from 56 patients and blood donors. The RBC-FluoGene ABO Basic kit uses SSP-PCR with fluorescence readout to differentiate between the five target common alleles, *A, A2, B, O1,* and/or *O2.* It takes 90 minutes to complete, and

genotype results are generated by the accompanying software. The PCR-RFLP method, though involving more hands-on time than the commercial TaqMan-based SSP-PCR test, is lower cost and involves the use of supplies typically found in a molecular laboratory. The main difference between the two assays is that the SSP-PCR assay defaults to assigning an *ABO*A* allele when positive amplification of all reactions associated with wild-type alleles ("non" reactions) is observed in conjunction with a lack of amplification of allele-specific (*A2, B, O1,* or *O2*) "positive" reactions, whereas the PCR-RFLP assigns *ABO*A1* when the sample is positive for three particular SNVs (467C, 703G and 1096G; Table 3). Although the differentiation between A and A2 for purposes of blood transfusion may be minimal, differentiating A1 from A2 for purposes of solid organ transplant is more significant.[6]

Sanger sequencing blood group antigen genes is considered the gold standard when resolving serologic typing or serologic-genotype discrepancies. Unlike TaqMan-based SSP-PCR and PCR-RFLP, Sanger sequencing of the exons and exon-intron borders of the *ABO* gene can detect genetic variation when compared with the reference sequence, and therefore is more sensitive at detecting alleles encoding subgroups. In this sample set, sequencing was used to identify the presence of rare group O or A subgroups. Of note, all 16 samples in this study that carried a group B allele gave concordant results between the two test methods and did not necessitate further sequence analysis. Sequencing and sequence alignments can be labor-intensive and if multiple SNVs are found, allele assignments can be equivocal. In this study, 7 of the 56 samples tested using

Table 3. *ABO* alleles *A1, A2, B, O1,* and *O2:* the genetic variants that are associated with them and those that are interrogated by each of the two methods

Common allele	Reference SNVs according to ISBT Blood Group Allele table	Allele detection by PCR-RFLP*	Allele detection by TaqMan-based SSP-PCR
A1	Reference allele	467C, 703G, 1096G	261G, 802G, 803G, 1061C
A2	c.467C>T; c.1061delC	703G, 1096G	1061delC, 261G, 802G, 803G
B	c.297A>G; c.526C>G; c.657C>T; c.703G>A; c.796C>A; c.803G>C; c.930G>A	467C	261G, 802G, 803C, 1061C
O1	c.261delG	261delG, 467C, 703G, 1096G	261delG, 802G, 803G, 1061C
O2	c.53G>T; c.220C>T; c.297A>G; c.526C>G; c.802G>A	467C, 703G	802A, 261G, 803G, 1061C

*Fragment pattern expected by known mutation sites cleaved by restriction enzymes.

SNV = single nucleotide variant; ISBT = International Society of Blood Transfusion; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; SSP = sequence-specific primer.

the low-resolution methods TaqMan-based SSP-PCR and PCR-RFLP yielded discordant results and Sanger sequencing was used to determine the alleles and predict the phenotype (Table 2).

Of the 56 samples tested, three yielded an atypical banding pattern by agarose gel electrophoresis of the PCR-RFLP reaction. These three samples were predicted to be *A/O1* by TaqMan-based SSP-PCR (Table 2). Sanger sequencing yielded allele assignments of two of the three consistent with this prediction (cases 2 and 3). All three samples were found to carry rare O variant alleles, which explains the uncommon banding pattern on the PCR-RFLP. In case 1, however, no explanation was found to cause a non-*O1* reaction to amplify in the TaqMan-based SSP-PCR and result in an *ABO*A* prediction. The *ABO*O1.01.09* allele carries the c.261delG, resulting in a frameshift and a nonfunctional enzyme. Based on this allele assignment, the predicted phenotype for this patient is group O. An uncommon banding pattern result from PCR-RFLP can provide valuable information to prevent an inaccurate group A predicted phenotype and also to propose the presence of a variant allele necessitating sequence analysis.

Of the 56 samples tested, three (cases 4–6) were predicted to be *A/O1* by SSP-PCR and *A2/O1* by PCR-RFLP. Sequencing analysis resolved the discrepancy by identifying rare alleles containing the c.467T variant: two *ABO*IVS* alleles and one group O subgroup. A limitation of the PCR-RFLP is its use of the c.467 SNV to differentiate between *A1* and *A2*. Ethnicity could be considered to prompt additional testing when an *A2* genotype is assigned from the PCR-RFLP. For example, the c.467 SNV is commonly associated with the *ABO*A1.02* in individuals of Asian descent.[7]

It is important to note that there is no licensed molecular test for predicting the ABO group. Therefore, though genotyping can be a useful tool in discrepancy investigations, the results cannot be used to label a blood product. Further, serologic methods such as use of lectins can be used to resolve some discrepancies.[3,4]

Conclusions

Because of the complexity of the ABO blood group system and the limitations of these two platforms, experienced molecular immunohematologists are a useful resource when interpreting *ABO* genotyping results. The use of SNV genotyping to predict an ABO phenotype requires consideration of the platform used, an understanding as to which SNVs are detected, and the algorithm used to assign alleles. In addition, low-resolution SNV genotyping that leads to an *A1* result or a result that is not consistent with the serologic results should be considered with caution because rare *ABO* variants cannot be excluded.

Acknowledgments

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Jessica A. Keller, MS, MB(ASCP) (corresponding author), Consultant, Technical Trainer, National Molecular Laboratory, American Red Cross Biomedical Services, 700 Spring Garden Street, Philadelphia, PA 19123, Jessica.Keller@redcross.org; Trina Horn, MT, MS, SBB (ASCP), Director, Laboratory Services Product Development and Implementation, American Red Cross Biomedical Services, Philadelphia, PA; Sabine Scholz, Product Manager, inno-train Diagnostik, Kronberg, Germany; Sabrina Koenig, PhD, Product Manager, inno-train Diagnostik, Kronberg, Germany; and Margaret A. Keller, PhD, Senior Director, National Molecular and Genomics Laboratories, American Red Cross Biomedical Services, Philadelphia, PA.

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