Determination of ABO blood group genotypes using the real-time loop-mediated isothermal amplification method

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Received October 31, 2014; Accepted June 17, 2015

DOI: 10.3892/mmr.2015.4144

Abstract. ABO genotyping is commonly used in several situations, including blood transfusion, personal identification and disease detection. The present study developed a novel method for ABO genotyping, using loop-mediated isothermal amplification (LAMP). This method allows the simultaneous determination of six ABO genotypes under 40 min at a constant temperature of 62˚C. The genotypes of 101 blood samples were determined to be AA (n=6), AO (n=38), BB (n=12), BO (n=29), AB (n=8) and OO (n=8) by the LAMP assay. The results were compared with the phenotypes determined by serological assay and the genotypes determined by direct sequencing, and no discrepancies were observed. This novel and rapid method, with good accuracy and reasonably cost effective, provides a supplement to routine serological ABO typing and may also be useful in other point-of-care testing.

Introduction

The ABO blood group, which was discovered at the beginning of the 20th century by Karl Landsteiner remains the most important blood group clinically (1-3). In 1924, this blood group was classified into four antigens (A, B, O and AB) and six genotypes (AA, AO, BB, BO, OO and AB). It is one of the conventional blood group polymorphisms, which are important for genetic markers in linkage analysis, blood transfusion, personal identification and disease detection (4,5).

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Key words: ABO blood group, genotyping, real-time loop-mediated isothermal amplification

A range of techniques have been used for ABO genotyping, including polymerase chain reaction (PCR)-restriction fragment length polymorphism, PCR-single-strand conformation polymorphism, allele-specific-PCR, PCR-amplified product length polymorphism, reverse transcription-quantitative (RT-q)PCR and DNA chip (3,6-8). These methods are, however, time-consuming and disadvantageous due to the requirement for labor-intensive post-amplification procedures, including restriction electrophoresis and enzyme cleavage, or the use of radioactive-labeled DNA probes. Therefore, a simpler, quicker and more informative method for the genotyping of the ABO alleles is required.

The present study aimed to improve the ABO genotyping method, based on LAMP, which directly uses a one-step isothermal reaction to determine the above-mentioned six genotypes. The developed technique was a powerful innovative gene amplification approach as a simple rapid tool for clinical detection and identification.

Materials and methods

Samples and DNA extraction. Peripheral blood samples were collected from 101 unrelated Chinese volunteers into EDTA-coated tubes at the Shaanxi Provincial People's Hospital (Xi'an, China) with informed consent. The study was approved by the ethics committee of the College of Life Sciences, Northwest University (Xi'an, China). The ABO phenotypes of all blood samples were identified by serological methods, based on the ABO blood group antigens present on red blood cells and IgM antibodies present in the serum. The genomic DNA from the volunteers was isolated from 1 ml blood using a Whole Blood Genomic DNA Isolation kit (Xi'an GoldMag Nanobiotech Co., Ltd., Xi'an, China), according to the manufacturer's instructions.

Primer design and synthesis. Two single nucleotide polymorphisms (SNPs) at nucleotides 261 and 803, located on exons 6 and 7 of the ABO gene, which cover the most polymorphic sites of the complete ABO sequences, as shown in Fig. 1 (9), were selected. The primer set for LAMP amplification includes a set of four primers, comprising two outer and two internal primers, which recognize six distinct regions on the target sequence, designated in Fig. 1. Table I lists the

Table I. Sequences of typing primers used for real-time loop-mediated isothermal amplification reaction.

Allele	Primer	Sequence (5'→3')
nt 261 for O test	O-F3	TGTGCCAGAGGCGCAT
	O-B3	TGATGGCAAACACAGTTAAC
	O-FIP	TACCACGAGGACATCCTTCCTCCTGCCAGCTCCATGTGA
	O-BIP	ACCCCTTGGCTGGCTCCTGGAGCCTGAACTGCTCGT
	Non-O-FIP	CACCACGAGGACATCCTTCCTCCTGCCAGCTCCATGTGA
	Non-O-BIP	GACCCCTTGGCTGGCTCCTGGAGCCTGAACTGCTCGT
nt 803 for B test	B-F3	TGACTGGTTCGGCACCCT
	B-B3	TGCCGTTGGCCTGGTCGAC
	B-FIP	TGTAGTAGAAATCGCCCTTCACGGAAGCAGCCGGGA
	B-BIP	CGTTCTTCGGGGGGTCGGTCCGGTACTACCA
	Non-B-FIP	GGTAGTAGAAATCGCCCTTCACGGAAGCAGCCGGGA
	Non-B-BIP	GGTTCTTCGGGGGGTCGGTCCGGTACTACCA

Specific nucleotides are underlined. nt, nucleotide; FIP, forward inner primer; BIP, backward inner primer.

Table II. Possible patterns detected with real-time loop-mediated isothermal amplification and statistical results of genotyping of the 101 selected individuals.

Phenotype	Genotype	B primer set	non-B primer set	O primer set	non-O primer set	No. individuals identified (n=101)	Calculated genotype frequency (%)
A	AA	-	+	-	+	6	5.94
A	AO	-	+	+	+	38	37.62
B	BB	+	-	-	+	12	11.88
B	BO	+	+	+	+	29	28.71
AB	AB	+	+	-	+	8	7.92
O	OO	-	+	+	-	8	7.92

sequences of the typing primers used for the LAMP reaction. All oligonucleotide primers were synthesized by Beijing Genomics Institute (BGI; Beijing, China).

Target amplification by LAMP. Each analysis was performed by subjecting samples of prepared blood from each individual to four LAMP amplifications. Two reactions used the O or B primer set and the other reactions used the non-O or non-B primer set. Each reaction included a forward/backward primer (F3/B3). The amplification was performed in a final volume of 25 μl, containing 0.8 μM forward inner primer and backward inner primer, 0.2 μM F3 and B3 primers, 15 μl Isothermal Master mix (IMM; OptiGene, Horsham, UK) and 50 ng genomic DNA. The reaction conditions were optimized to ensure that the amplifications were highly specific, including assessment of primer concentration, preparation blood concentration and reaction temperature. The LAMP assays were performed in 8-well strips by incubation at 62˚C for 40 min. Simultaneously, the fluorescence intensity of the florescent dye, contained in the IMM, was monitored using a Genie II system (OptiGene).

Direct sequencing of the PCR products. For the samples to be sequenced, a 200 bp fragment for exon 6 and a 740 bp fragment for exon 7 were amplified using the following two primer pairs synthesized by BGI: Exon 6, forward: 5'-TCCATGTGACCG CACGCCTC-3' and reverse: 5'-GGGTCTCTACCCTCGGCC ACC-3'; exon 7, forward: 5'-CCGTGTCCACTACTATGTCTT CACC-3' and reverse: 5'-ACAACAGGACGGACAAAGGAA ACAG-3'. The PCR products were sequenced by the BGI.

Results and Discussion

Using this method, base substitutions at two SNP sites in the ABO gene (nucleotides 261 and 803) were detected simultaneously by four primers, setting the extension reaction to distinguish the six genotypes (AA, AO, BB, BO, OO and AB). In the case of haplotypes, a positive allele-specific reaction excluded the corresponding positive non-allele-specific reaction and vice versa. Allele B was distinguished from the non-B primer at nucleotide 803 and allele O was distinguished from the non-O at nucleotide 261. A summary of all possible patterns are indicated in Table II and representative data are shown in Fig 2.

A pool of 101 blood samples from Chinese donors was assessed using LAMP. The detected genotypes, together with their frequencies, are shown in Table II. The observed allele frequencies were: 28.71, 30.20 and 41.09%, for A, B and O,

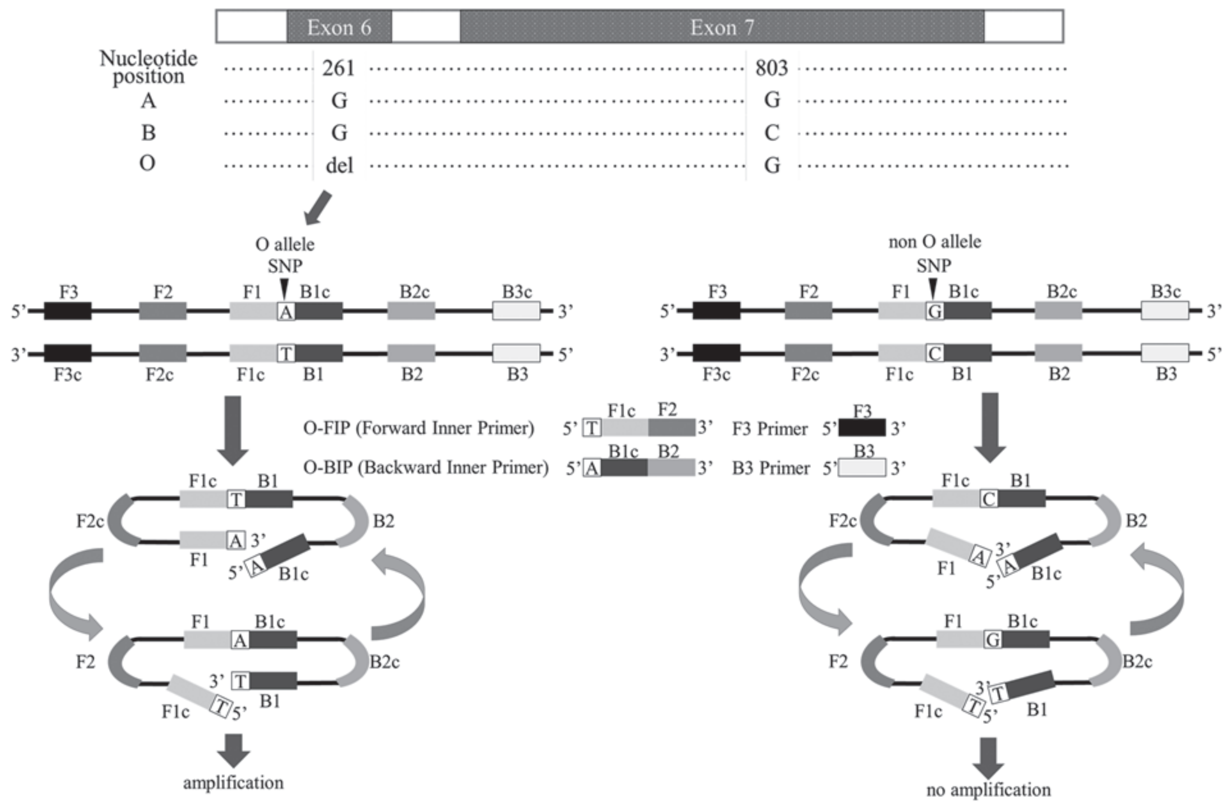

Figure 1. Schematic of primers and the process for real-time loop-mediated isothermal amplification using the O primer set used as an example.

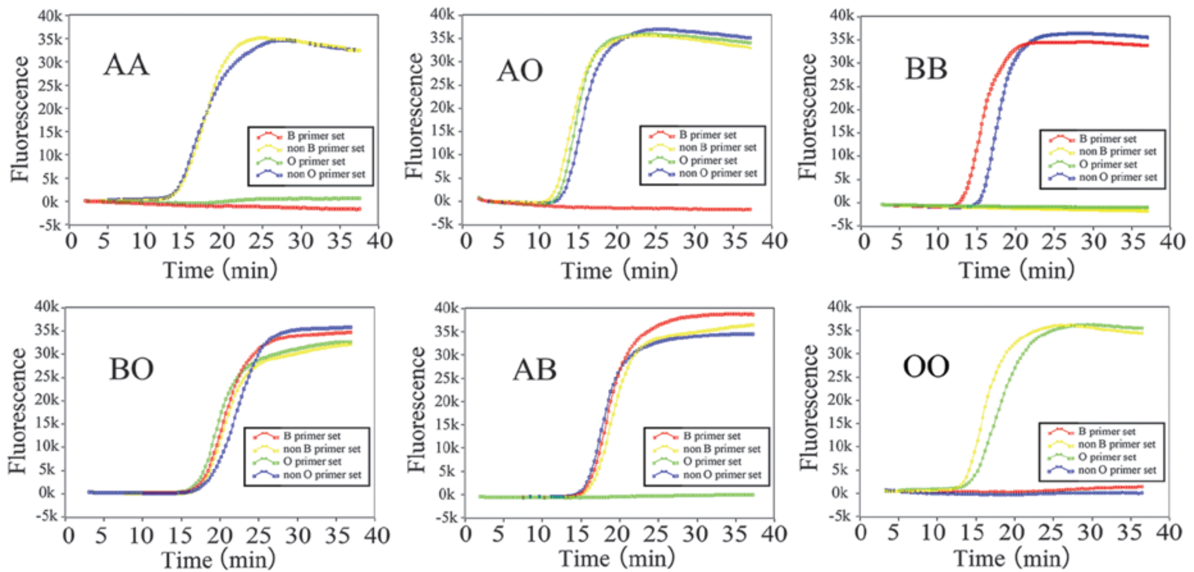

Figure 2. Real-time loop-mediated isothermal amplification-based detection of the ABO blood group genotype. The following genotypes were identified: AA, AO, BB, BO, AB and OO.

respectively (Calculated from: A: (AA+AO/2+AB/2)/101; B:(BB+BO/2+AB/2)/101; O: (AO/2+BO/2+OO)/101), from which phenotype frequencies were calculated to be 43.56, 40.59, 7.92 and 7.92% for A, B, AB and O, respectively (Calculated from: Phenotypes/101). The results were compared with the phenotypes determined by serological assay and the genotypes determined by direct sequencing, and no discrepancies were observed.

The ABO blood group system was the first blood group system to be identified and has been used extensively as a marker in population studies, epidemiology, and forensic investigation (10). The ABO blood group is fundamental for transfusion medicine, as accurate testing of the blood donor and recipient is essential for the prevention of hemolytic transfusion reactions and hemolytic disease of the newborn (11,12). The ABO blood group is also critical for

assessing the risk of developing certain malignancies (13,14) and cardiovascular disorders (5,15). To date, several methods have been reported for ABO genotyping, which rely predominantly on differentiating A, B and O at specific base substitutions.

However, technical errors and several clinical conditions or diseases can lead to discrepancies between erythrocytes and sera, resulting in an incorrect genotype being determined (3,16-18). An emerging novel approach, LAMP, is gaining attention as a result of its practicality, speed and usefulness in laboratories and clinical settings (19-21).

LAMP is a simple, rapid and accurate gene amplification technique, using a set of four specially designed primers to span six distinct regions on the target gene. The amplification procedure is simple and rapid, wherein the whole procedure can be completed in a single step by incubating all reagents (samples, primers, DNA polymerase with strand displacement activity and substrates) in a single tube under isothermal conditions, which can be completed in <1 h, with the DNA being amplified 10^{-9}-10^{-10} times (22).

The primary characteristic of LAMP is its advantage of reaction simplicity and higher amplification efficiency. In the present study, the DNA amplification procedure was <40 min at a constant temperature of 62˚C, which allowed no time loss for thermal change. The DNA polymerase provides high amplification efficiency as a result of its high specificity and the presence of the target gene sequence is easily detected by judging the presence of amplified products. In addition, no denaturation of double stranded DNA into a single stranded DNA is required. In addition, the more sensitive signal detector instrument, GENIE II, assists in the rapid processing of the data. It takes ~20 min to reach the peak, followed by another 20 min to complete the entire reaction, which is relatively quicker compared with normal PCR-based assays.

The second characteristic of LAMP is its superior specificity. It has been previously reported that the detection limit of the LAMP assay was 10 times lower compared with RT-qPCR and 10^4 times lower compared with conventional PCR (22,23). However, LAMP systems with high sensitivity may lead to false positive results. In the present study, the blood samples from 101 Chinese individuals were successfully genotyped using peripheral blood DNA and no false positive reaction was observed, according to the direct sequencing.

With the use of four primers designed to recognize six distinct regions, only the target SNP is strictly and specifically amplified, even in coexistence with its homologous gene. The reaction is so specific that it can strictly discriminate single nucleotide differences. These results demonstrated that the four primer sets designed were extremely specific to the two SNP sites in the ABO gene. Also, the application of IMM, including florescent dye, reduced the risk of cross contamination due to the reduction of lid opening.

In conclusion, the LAMP assay developed in the present study has great potential for rapid ABO genotyping, which can be applied in laboratories and clinical settings. It is also envisioned that several other known SNPs may also be detected by this method with corresponding primer sets. Considering the advantages of rapid amplification, easy detection and simple operation, LAMP may offer more applications for point-of-care testing.

Acknowledgements

The present study was supported by the Project of National Great New Drug Research and Development of China (no. 2012ZX09506001-001) and a grant from the National Institute of Health (no. P20RR016457 from the INBRE Program of the National Center for Research Resources).

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