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Direct determination of ABO blood group genotypes from whole blood using PCR-amplification of specific alleles method

Kensaku Aki[1], Kazuyoshi Kawazoe[2], Azusa Izumi[3], Tomoki Tada[1, 4], Kazuo Minakuchi[2], Eiji Hosoi[1]

[1]Department of Cells and Immunity Analytics, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan
[2]Department of Pharmacy, Tokushima University Hospital, Tokushima, Japan
[3]Clinical Laboratory, Mie Prefectural Shima Hospital, Mie, Japan
[4]Subdivision of Biomedical Laboratory Sciences, Graduate School of Health Sciences, The University of Tokushima, Tokushima, Japan

Email address:
aki@medsci.tokushima-u.ac.jp (K. Aki), hosoi@medsci.tokushima-u.ac.jp (E. Hosoi)

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Abstract: ABO antigens are known to be oligosaccharide antigens, and are widely expressed on the membranes of red blood cells and tissue cells. Therefore, the determining ABO blood group antigens is important in both transfusion and organ transplantation, and is one of the markers used for personal identification in forensics. The ABO blood group is currently determined by the presence of A and B antigens on red blood cells using serological tests in clinical laboratories. The gene sequences of the ABO blood group in chromosome 9q34.1-q34.2 have also been determined. Accordingly, it has become possible to genetically analyze the ABO blood group using molecular biological techniques. We recently developed an ABO genotyping method based on PCR amplification of specific alleles (PASA) using DNA extracted from blood and saliva. However, the extraction and purification of DNA is necessary prior to PCR because blood and saliva contain various substances that inhibit PCR. Furthermore, the PCR amplification of specific alleles (PASA) method requires specificity and stability for allele-specific amplification. Therefore, it is very difficult to use whole blood directly. Here, we described the development and use of ABO genotyping from whole blood using a commercially available reagent kit, which can effectively neutralize inhibitory substances present in the blood. In this study, for all genes of the six major ABO genotypes and cisA2B3 genotype of the AB variant, only specific bands were clearly amplified, whereas non-specific bands were not amplified at all. In addition, this method was able to determine ABO genotyping using 5-fold diluted fresh whole blood, or 5-fold diluted whole blood that was freeze-stored in 100 μL aliquots at -20 °C by subdivided for a maximum of 30 days. This analysis method to determine ABO blood group genotyping is simple and useful, and is expected to be used widely throughout research and clinical laboratories and forensic fields.

Keywords: ABO Blood Group, Direct PCR, DNA Typing, PASA: PCR Amplification of Specific Alleles, CisAB

1. Introduction

The ABO blood group is the one of the most important among the 30 blood group systems. After the discovery of ABO antigens on red blood cells from serological differences in human blood by Landsteiner in 1900, the ABO blood group was classified into four antigens (A, B, O, and AB) and six genotypes (*A/A, A/O, B/B, B/O, O/O,* and *A/B*) in 1924 [1-4]. Furthermore, biochemical characteristics of the ABO antigens and structure of the ABO gene were elucidated in numerous studies [5-9].

Genes of the ABO blood group have been determined at chromosome 9q34.1-q34.2 [10-13], and Yamamoto et al. [10, 11] cloned and determined these structures in 1990. These findings have made it possible to genetically analyze ABO blood group antigens using molecular biology techniques [14-21]. Therefore, genetic analysis of the ABO blood group has become important in both transfusion and organ

transplantation, and is used as one of markers for personal identification in forensics.

We developed an ABO genotyping method based on PCR amplification of specific alleles (PASA) using DNA extracted from blood and saliva [22]. This PCR amplification of specific alleles (PASA) method allows for the simple and rapid detection of multiple single nucleotide polymorphism (SNP) sites on the ABO gene, and is useful for determining the ABO genotype. However, this PCR amplification of specific alleles (PASA) method requires DNA extraction and purification, and PCR conditions for the specificity and stability of allele-specific amplification, because some substances in blood and saliva may interfere with the activity of enzymes such as thermostable DNA polymerase. These substances inhibit PCR reaction and make it difficult to obtain valid results, so it is difficult to use whole blood directly. Previous studies have investigated PCR methods that do not require prior DNA isolation, however, these methods have limitations. For example, some methods require a particular DNA polymerase such as a Tth DNA polymerase. Other methods require the pretreatment or heat-treatment of the sample, which is time-consuming [23-27].

Therefore, it is very difficult to directly use whole blood for the PCR amplification of specific alleles (PASA) method. However, a novel reagent has recently been developed, that can neutralize the various substances present in the blood that inhibit PCR.

In this study, we attempted to improve the ABO genotyping method based on PASA from whole blood without extracting DNA using a commercially available reagent kit.

2. Materials and Methods

2.1. Ethics Statement

The study was approved by the Ethics Committee for Human Genome/Gene Research of the University of Tokushima. Written informed consent was obtained from all volunteers, and all participants signed the Ethics Committee-approved consent forms.

2.2. Subjects

Whole blood samples (7 genotypes: *A/A, A/O, B/B, B/O, O/O, A/B,* and *A2B3/O*) were collected using EDTA-2K from healthy volunteers who had previously been typed serologically and genotyped.

2.3. Design of Allele-Specific PCR Primers for ABO Genotyping

The allele-specific primer set distinguishes four SNPs (nucleotides 261, 526, 796, and 803) from the ABO gene to identify the A, B, O, and cisAB alleles. Table 1 shows a summary of the primer combinations, oligonucleotide primer sequences, product band size, and allele specificities. The 3' endbase of primers (1), (3), (6), (7), and (9) and (10) corresponded to the nucleotide sequences of O, A and B, B, A and O, and cisAB alleles, respectively. Primers (2), (4), (5), and (8) corresponded to the nucleotide sequence of the ABO common allele. Primers (6), (9) and (10) were inserted into a single artificial mismatched nucleotide (T) at the second nucleotide (C) from the 3' end of primers to improve the specificity and stability of allele-specific amplification from whole blood samples.

2.4. ABO Genotyping by Polymerase Chain Reaction Amplification of the Specific Allele (PASA) from Whole Blood

The PASA reaction distinguishes different ABO genotypes on the basis of the molecular size of allele-specific amplification products that contain 261, 526, 796, and 803 nucleotides (the sites of amino acid substitutions) in ABO allelic cDNA. Details of this procedure and validation of the PASA method for ABO genotyping have been reported elsewhere [16-18, 22]. The scheme of the amplification method and analysis of specific ABO alleles using the PASA method are shown in Figure 1, and a summary of all possible specific bands of ABO and cisAB genotype patterns obtained with the PASA method are given in Table 2.

2.5. PCR Conditions

PCR 1 and 2 amplifications using the two sets of primers were carried out in a final volume of 20 µl containing 1 µl of a 5-fold dilution of fresh whole blood with sterilized ultrapure water (5-fold dilution of fresh whole blood), 50 pmol of each primer, 0.1 µl of BIOTAQTM HS DNA polymerase (BIOLINE), and 10 µl of 2×Ampdirect® Plus (Shimadzu Corp, Kyoto, Japan) containing $MgCl_2$ (3mM) and dNTPs (400µM each). PCR 3 amplification using the two sets of primers were carried out in a final volume of 20 µl containing 2 µl of the 5-fold dilution of fresh whole blood with sterilized ultrapure water, 100 pmol of each primer, 0.1 µl of BIOTAQ HS DNA Pol (BIOLINE), and 10 µl of 2×Ampdirect® Plus (Shimadzu Corp, Kyoto, Japan) containing $MgCl_2$ (3mM) and dNTPs (400µM each). PCR 1, 2, and 3 amplification conditions were an initial denaturation step at 95 ^{o}C for 10 minutes, followed by 37 cycles of denaturation at 95 ^{o}C for 30 sec, annealing at 67 ^{o}C for 30 sec, and extension at 72 ^{o}C for 30 sec, and the last cycle extension was performed for 7 min at 72 ^{o}C, followed by a quick chill to 4 ^{o}C. The PCR product was mixed with a loading buffer gel and was then applied to a 3% agarose gel (Wako, Osaka, Japan) that contained 0.5 µg/ml of ethidium bromide. Electrophoresis was visualized with Printgraph (ATTO, Tokyo, Japan).

2.6. Optimal Preparation and Storage Conditions of Whole Blood for ABO Genotyping

To determine optimum ABO genotyping from whole blood, three different preparation and storage conditions of whole blood were carried out. Moreover, we clarified the

influence on the PCR reaction by the storage of whole blood.

Under the first set of conditions, whole blood was stored at 4°C after collection (0 days), 2, 5, and 30 days, and was used directly or after a 5-fold dilution of whole blood with sterilized ultrapure water (5-fold dilution of whole blood) at the time of the experiment. Under the second set of conditions, whole blood was diluted 5-fold with sterilized ultrapure water immediately after collection, was frozen at -20 °C, and was then used as a 5-fold dilution of whole blood that was repeatedly frozen and thawed 2 days, 7 days, and 14 days later in each experiment. Under the last set of conditions, whole blood was diluted 5-fold with sterilized ultrapure water immediately after collection, was subdivided into four aliquots of 100 μL, stored at -20 °C for 3 days, 7 days, 30 days, and 180 days, and was then used directly after thawing. These experiments were carried out by only changing the preparation and the storage conditions without changing the PCR conditions.

Table 1. *Allele-specific primer sets used in this study*

No.	Primer	Primer Sequence (5'→3')	Product size(bp)	Allele specificity
(1)	O-261N	CAGTAGGAAGGATGTCCTCGTGGTA	118	O allele
(2)	ABO-1R3	CTTCTTGATGGCAAACACAGTTA		
(3)	ABO-1FAB2	GAAGGATGTCCTCGTGGTG	133	A, B allele
(4)	ABO-1R	TTAACCCAATGGTGGTGTTCTGG		
(5)	fy-43	GGATCCAGGGGTGCACGGCCGGCGGC	224	B allele
(6)	526-B2	CTGCCAGCGCTTGTAGGCGTC		
(7)	526-A	CAGCTGTCAGTGCTGGAGGTGC	379	A, O allele
(8)	Fy-2	CCGTTGGCCTGGTCGACCATCATGGCCTG		
(9)	796-A2	AAGGACGAGGGCGATTTCTACTATC	55	cisAB allele
(10)	803-B2	TCTTGCACCGACCCCCCGAAGAATG		

The 3' endbase of primers (1), (3), (6), (7), and (9) and (10) corresponded to the nucleotide sequences of O, A and B, B, A, and O, and cisAB alleles, respectively. Primers (2), (4), (5), and (8) corresponded to the nucleotide sequence of the ABO allele. Primers (6), (9), and (10) were inserted at an artificial mismatched nucleotide at the second nucleotide from the 3' end of primers. *: Primers (5) and (8) were quoted from reference 15, 14, respectively.

Table 2. *Patterns of the PASA method for all possible ABO genotypes*

							cisAB		
	A/A	*A/O*	*B/B*	*B/O*	*O/O*	*A/B*	A_2B_3/O	A_1B_3/A_1	A_2B/B
379 (A, O specific)	+	+	−	+	+	+	+	+	+
224 (B specific)	−	−	+	+	−	+	−	−	+
133 (O specific)	−	+	−	+	+	−	+	−	−
118 (A, B specific)	+	+	+	+	−	+	+	+	+
55 (cisAB specific)	−	−	−	−	−	−	+	+	+

+, Presence of the expected specific ABO gene-type fragment. −, Absence of the expected specific ABO gene-type fragment. (a), Specific band of the A and O alleles using primers (7 and 8). (b), Specific band of the B allele using primers (5 and 6). (c), Specific band of the O allele using primers (1 and 2). (d), Specific band of the A and B alleles using primers (3 and 4). (e), Specific band of the cisAB allele using primers (9 and 10).

3. Results

3.1. ABO Genotyping by the PASA Method using Whole Blood

In this study, the PASA reaction was used to distinguish the different ABO genotypes of genomic DNA in fresh whole blood samples donated from volunteers on the basis of the molecular size of allele-specific amplification products containing 261, 526, 796, and 803 nucleotides (the sites of amino substitution) in ABO allelic cDNA.

As shown in Figure 2, all genes of the six major ABO genotypes, *A/A, A/O, B/B, B/O, O/O,* and *A/B* were amplified using the 5-fold dilution of fresh whole blood immediately after direct collection; two specific bands (379 and 118 bp) for *A/A*, three specific bands (379, 133, and 118 bp) for *A/O*, two specific bands (224 and 118 bp) for *B/B*, four specific bands (379, 224, 133, and 118 bp) for *B/O*, two specific bands (379 and 133 bp) for *O/O,* and three specific bands (379, 224, and 118 bp) for *A/B*. Only the specific bands were amplified clearly for all genotypes, whereas non-specific bands were not amplified at all.

Figure 3 shows patterns of amplification of the typical types of *A/O, O/O,* and *cisA2B3/O*. The *cisA2B3/O* genotype was established by four specific bands (379, 133, 118, and 55 bp) of three PCRs (PCR-1, 2, and 3). The 55 bp band in PCR-3 was a specific band in the cisA2B3 allele.

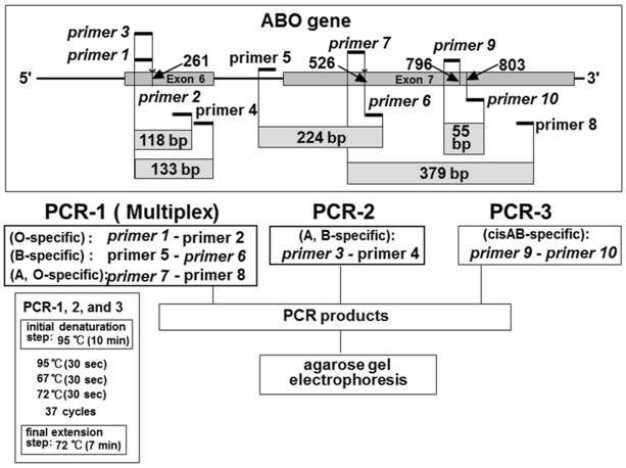

Figure 1. *Scheme of amplification and analysis of specific ABO alleles using the PASA method. Allele-specific DNA fragments of the O allele (133 bp), A and B alleles (118 bp), B allele (224 bp), A and O alleles (379 bp), and cisAB allele (55 bp) were amplified by PCR with five primer pairs (primers 1 and 2, primers 3 and 4, primers 5 and 6, primers 7 and 8, and primers 9 and 10, respectively). Two (118 and 379 bp) of the five fragments were co-amplified in a single PCR 1 (multiplex-PCR). Two (133 and 224 bp) of the five fragments were co-amplified in a single PCR 2 (multiplex-PCR). Primers 6, 9, and 10 were inserted at an artificial mismatched nucleotide at the second nucleotide from the 3′ end of primers.*

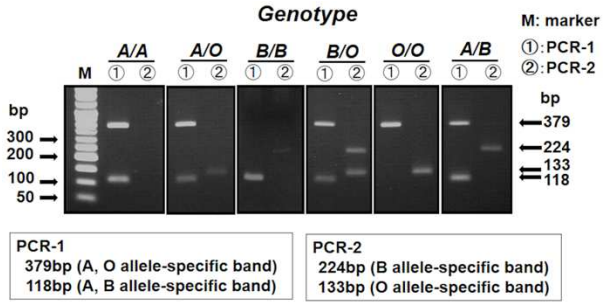

Figure 2. *Direct PCR from whole blood of the six major ABO genotypes. Direct PCR was carried out using a 5-fold dilution of fresh whole blood by the PASA method using 4 primer sets [①, PCR 1 (primers 1 and 2, and primers 7 and 8), ②, PCR 2 (primers 3 and 4, and primers 5 and 6)]. M, 50 bp DNA size marker. The six major ABO genotypes were of A/A, A/O, B/B, B/O, O/O, and A/B.*

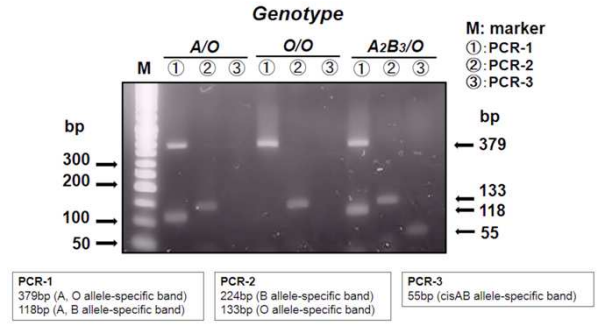

Figure 3. *Direct PCR from whole blood of cisAB (A2B3/O). Direct PCR was carried out using a 5-fold dilution of fresh whole blood by the PASA method using 5 primer sets [①, PCR 1 (primers 1 and 2, and primers 7 and 8), ②, PCR 2 (primers 3 and 4, and primers 5 and 6), ③, PCR 3 (primers 9 and 10)]. M, 50 bp DNA size marker. The electrophoresis results of A/O and O/O are showed at the same time in order to compare them with those of A2B3/O.*

3.2. Influence on ABO Genotyping of the Preparation and Storage Conditions Used for Whole Blood

Whole blood was stored under several conditions. Figure 4 shows a the comparison of the results of specific *A/B* and *O/O* bands obtained by direct PCR using 4°C stored whole blood or a 5-fold dilution of 4°C stored whole blood for 0 days, 2 days, 5 days, and 30 days.

In the analysis using a 5-fold dilution of 4°C stored whole blood after 0 days and 2 days, only specific bands were amplified, whereas non-specific bands were amplified after 5 days and 30 days; the 224bp band for type *O/O*, and the 133bp band for type *A/B* and the 118bp band for type *O/O*, respectively. A non-specific band of 118bp was very weak.

On the other hand, in the analysis using 4°C stored whole blood directly immediately after blood collection and 2 days later, specific bands were weakly and partially amplified, while only specific bands were amplified using whole blood after 5 days. Furthermore, in the analysis using whole blood after 30 days, a non-specific band of 118bp was amplified weakly for type *O/O*.

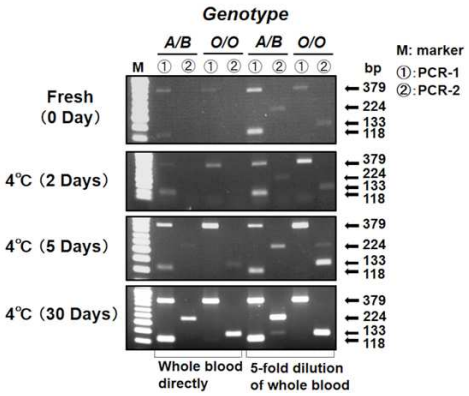

Figure 4. *Direct PCR from 4°C stored whole blood or 5-fold dilution of 4°C stored whole blood. The comparison of results of specific bands for A/B and O/O obtained by direct PCR using 4°C stored whole blood or a 5-fold dilution of 4°C stored whole blood for after collection (0 days), 2, 5, and 30 days.*

Figure 5 shows a comparison of results for specific bands on direct PCR of typical types *A/B* and *O/O* using the 5-fold diluted whole blood that was stored for 0 days, 2 days, 7 days, and 14 days at -20°C, and was repeatedly frozen and thawed in each experiment. Although only specific bands were clearly amplified in the analysis using whole blood immediately after blood collection, 2 days, and 7 days, a non-specific band was amplified 14 days later; the 224bp band for type of *O/O*. This non-specific band were very weak, but this band was expected to be stronger with time from the other results in this study.

Figure 6 shows the results of specific bands on direct PCR of typical types *A/B* and *O/O* using the 5-fold diluted whole blood that was stored for 3 days, 7 days, 30 days, and 180 days at -20°C by subdivision, and was used directly after thawing. Although a specific band was only clearly amplified in the analysis using the 5-fold diluted whole blood that was thawed after 3 days, 7 days, and 30 days, a

non-specific band of 133bp was amplified in type AB after 180 days. This non-specific band could be excluded completely by reducing PCR cycles to 35 cycles from 37 cycles. Reducing the number of PCR cycles allowed non-specific amplification to also be reduced.

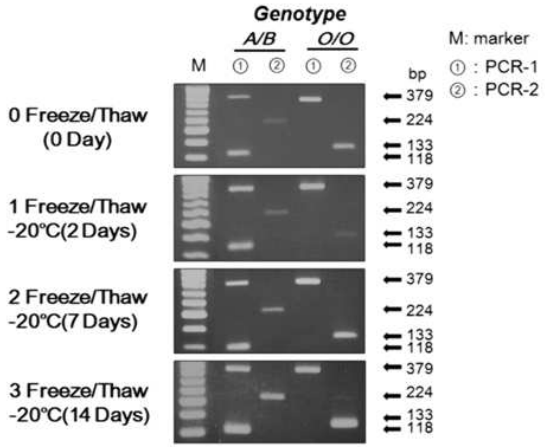

Figure 5. *Direct PCR from 5-fold diluted whole blood that was freeze-stored at -20°C. The comparison of results of specific bands for A/B and O/O obtained by direct PCR using a 5-fold diluted whole blood that was stored at -20 °C and a 5-fold diluted whole blood that was repeatedly frozen and thawed 2 days, 7 days, and 14 days later in each experiment.*

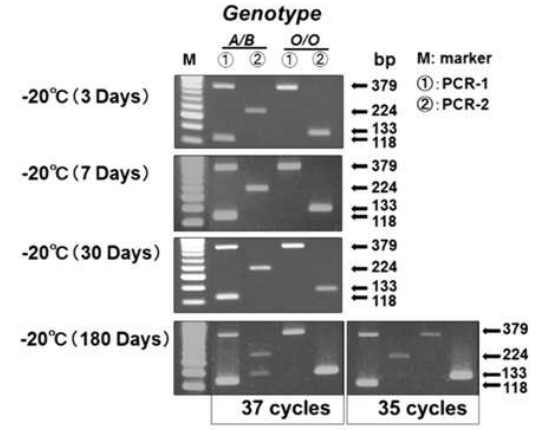

Figure 6. *Direct PCR from 5-fold diluted whole blood that was freeze-stored at -20°C by subdivision. The comparison of results of specific bands for A/B and O/O obtained by direct PCR using a 5-fold diluted whole blood that was subdivided and stored at -20 °C for 3 days, 7 days, 30 days, and 180 days, and was used directly after thawing. A non-specific band of 133 bp was amplified in type AB after 180 days could be excluded completely by reducing PCR cycles to 35 cycles from 37 cycles.*

4. Discussion

The gene sequences of the ABO blood group in chromosome 9 have recently been determined and it has become possible to detect ABO genotyping using molecular biology techniques. We previously reported the PASA method with DNA extracted from the blood and saliva. However, this method requires DNA extraction, and was shown to be time-and effort-consuming. We then attempted to establish an ABO genotyping method based on PASA method from whole blood without extracting the DNA. However, it is difficult to use whole blood directly for PASA because blood contains various substances that inhibit PCR, such as heme compound [28], immunoglobin G [29], other unknown inhibitors [30], and anticoagulants [31], which makes it difficult to obtain valid results. AmpDirect® plus reagent is said to be a novel reagent cocktail intended to neutralize various charge-bearing inhibitory substances that may otherwise bind to DNA polymerase and template DNA [32, 33].

On the other hand, a genotyping method based on PASA is a more rapid, simple, and useful tool for determining the genotype of the ABO blood group than the PCR-RFLP (Restriction Fragment Length Polymorphism), PCR-direct sequencing, and PCR-SSCP (Single Strand Conformation Polymorphism) methods, which require 2-step procedures. [11, 15, 16, 34-36] However, not only specific bands, but also non-specific bands are amplified easily by the PASA method. Furthermore, certain conditions are necessary when whole blood is directly used. Therefore, to improve the specificity of allele-specific amplification, we first designed allele-specific primers in which a single artificial mismatched nucleotide was inserted at the second nucleotide from the 3' end of primers such as the 526-B2, 796-A2 and 803-B2, determined the primer combination, pretreated the 5-fold dilution of whole blood, and determined other PCR conditions for multiplex-PCR. As a result, non-specific bands was suppressed, and it was possible to amplify only the specific band for the genotype of each blood type. Amplified band patterns were also consistent with the analysis results using DNA extracted from blood.

In addition, this PASA method is useful for analyzing the type cisAB (i.e., cisA1B3, cisA2B3, and cisA2B). CisAB alleles were shown to be identical to one another while being different from the A1 allele by two nucleotide substitutions (467C>T and 803G>C in exon 7), and the nucleotide substitution of 803G>C is a useful marker for the cisAB allele. This allele encodes both A and B transferase functions; however this transferase activity is very weak. On the other hand, although it is difficult to determine cisAB by a serological test alone, this PASA method can determine type cisAB quickly and easily. In the present study, we analysed subtype cisA2B3, which is one of the type cisABs.

Futhermore, we examined the influence of three different preparation and storage conditions of whole blood on ABO genotyping. The results showed that the storage and time at 4°C affected the components in whole blood and the 5-fold dilution of whole blood because the amplification of specific bands in each PCR became stronger with time, whereas non-specific bands were amplified. Furthermore, the specific band yield of PCR using the 5-fold dilution of whole blood was stronger than when whole blood was directly used, and only specific bands were clearly amplified until 2 days later (Fig.4). The PCR reaction may have become stronger because the PCR inhibitors contained in whole blood were gradually broken down with time or were reduced by dilution with sterilized ultrapure water. The PCR inhibitors contained in whole blood in particular are likely to

decrease quickly at 4°C.

On the other hand, PCR using whole blood that was freeze-stored at -20°C was thought to be more stable than that stored at 4°C; however non-specific bands were amplified similar to the results of storage at 4°C (date not shown). We also examined the results obtained with PCR using the 5-fold diluted whole blood that was freeze-stored at -20°C. The results showed that only specific bands were amplified clearly until 7 days (Fig. 5). From these findings, one of the causes for varying the components in the blood may have been freezing whole blood, and repeatedly freezing and thawing. On the other hand, the specificity of PCR using 5-fold diluted whole blood that was freeze-stored in 100μl aliquots at -20°C by subdivision was maintained up to 30 days later (Fig. 6). However, it was possible to completely exclude non-specific bands 180 days later by reducing PCR cycles to 35 cycles from 37 cycles. Therefore, it may be necessary to determine the relationship between non-specific bands and the number of PCR cycles in long-term storage samples of more than 30 days at -20°C.

In this study, we developed an analysis method of ABO genotyping from fresh whole blood without extracting DNA. Recently, a similar techniques for ABO genotyping by allele specific primers and direct PCR amplifications without DNA extraction are reporting [37, 38], but these articles often report only a summary of a simple detection methods of multiple SNP sites on the ABO gene. In contrast, we determined optimal conditions for ABO genotyping, and summarized the influence on the PCR amplification by preparation and the storage conditions of whole blood.

As a result, ABO genotyping was possible in 5-fold diluted fresh whole blood or 5-fold diluted whole blood that was freeze-stored in 100 μL aliquots at -20 °C by subdivided for a maximum of 30 days. Moreover, it was revealed that the amplification of the non-specific bands in each PCR became stronger along with the storage time of whole blood.

This data have not been analyzed sufficiently, it is considered that there is a need to continue the study of the influence by the storage of whole blood in future.

In conclusion, this PASA method to determine ABO blood group genotyping is simple and useful, and is expected to be used widely throughout research and clinical laboratories and forensic fields.

Conflict of interest

None of the authors have any interests in declared.

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