ORIGINAL

The evaluation of histo-blood group ABO typing by flow cytometric and PCR-amplification of specific alleles analyses and their application in clinical laboratories

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Abstract : ABO antigens are oligosaccharide antigens, and are widely distributed on red blood and tissue cells as well as in saliva and body fluid. Therefore, these antigens are important not only for blood transfusion, but also for tissue cell and organ transplantations. Also, blood, hair, and seminal fluid are important sources of evidence at crime scenes, and these antigens are some of the most important markers for personal identification in forensic investigations. Here, we describe the development and use of quantitative analysis of A, B, and H antigens on red blood cells by employing flow cytometric analysis and the ABO genotyping method based on PCR-amplification of specific alleles (PASA) within DNA, especially from blood and saliva. In this study, flow cytometric analysis could be used to compare the differences between the expression of A and/or B and H antigens on red blood cells with various phenotypes, and the PASA method was able to determine the genotype of the type cisA_2B_3 pedigree using only DNA extracted from saliva. These analysis methods are simple and useful for judging the ABO blood group system and genotyping, and are used widely throughout research and clinical laboratories and forensic fields. J. Med. Invest. 59 : 143-151, February, 2012

Keywords **:** *ABO blood group, flow cytometric analysis, cisAB, DNA typing, PASA : PCR amplification of specific alleles*

INTRODUCTION

After the discovery of ABO antigens on red blood cells in 1900 from serological differences in human blood (1), the ABO blood group was classified into four antigens (A, B, O, and AB) and six genotypes in 1924 (2-4). These antigens are oligosaccharide antigens, and are widely distributed on the membranes of red blood and tissue cells as well as in saliva and other body fluids. Recently, the nucleotide sequences of genes of the ABO blood group in chromosome 9q34, seven single base substitutions characterizing A compared with B and a single base deletion characterizing A compared with O, were determined (5-8). This has made it possible to analyze the ABO genotype using molecular biological techniques (9-16).

The A and B antigenic structures of the ABO blood group are defined as the carbohydrate determinants synthesized from the H precursor structure by the glycosyltransferase products of A and B genes, respectively (17). Therefore, the detection of ABO blood group antigens and genetic analysis of the ABO blood group are important in both

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transfusion and organ transplantation. Furthermore, the ABO blood group system is one of the most important markers of personal identification in forensics.

The classical method of detecting ABO antigens is the hemagglutination assay. This technique is simple, inexpensive, and is sufficient for routine ABO blood group typing in transfusion. However, hemagglutination, which is a qualitative assay, has certain limitations : it is difficult to quantify ABO antigens on red blood cells, and it does not precisely indicate the ABO blood type in normal or subgroups. In this study, we described the development and use of quantitative analysis of A, B, and H antigens by flow cytometric analysis and an ABO genotyping method based on PCR amplification of specific alleles (PASA) within DNA, especially from blood and saliva.

MATERIALS AND METHODS

Subjects

Peripheral blood samples (n=28, A type : 5, B type : 5, O type : 5, AB type : 5, cisA_1B_3 type : 2, cisA_2B_3 type : 5, A_2B type : 1) were collected from healthy volunteers who had previously been typed serologically and genotyped.

Preparation of glutaraldehyde-fixed spherical red blood cells

Blood anti-coagulated with EDTA-2K was centrifuged at 1,000× g for 10 min to separate the red blood cells from plasma, and washed three times in saline (1,000× g/2 min). Nine μL of packed red blood cells was added to 291 μL of saline, and then 1.5 ml of 0.11% glutaraldehyde fixing solution buffer containing 0.035 mM sodium dodecyl sulfate, 4.03 mM EDTA2Na, 3.36 mM EDTA4Na, and 109.3 mM NaCl (ADVIA 120 RBC/PLT reagent, Bayer Medical) for spheroidization and fixation was added, followed by incubation for 15 min at room temperature. It was then washed two times (1,000× g/2 min) with saline, and we finally prepared a 1.5% red blood cell saline suspension.

Flow cytometric analysis of A, B, and H antigens

Measurement of A and B antigens : Anti-human A and B mouse antibodies (Ortho-Clinical Diagnostics) were used as the first antibody and anti-Mouse IgM, H&L Chain Specific Fluorescein (FITC) Conjugate (Merck) as the second antibody. The negative control for the first antibody was a 1 : 30 dilution of Mouse IgM antibody (Dako). Fifty μl of a 1.5% red blood cell saline suspension was first incubated with 50 μl of a 1 : 64 dilution of the first antibody for 60 min at room temperature and, subsequently, washed two times (3,400 rpm/2 min) with saline, and then incubated again with 50 μl of a 1 : 100 dilution of the second antibody for 30 min at 4°C. It was then washed two times (3,400 rpm/2 min) and resuspended in saline, and analyzed employing FACS Calibur™ (Becton Dickinson).

Measurement of H antigen : Fifty μl of a 1.5% red blood cell saline suspension was first incubated with 50 μl of a 1 : 100 dilution of lectin from Ulex europaeus agglutinin-1 Fluorescein Conjugate (UEA-1, detecting H antigen) (Sigma) for 60 min at 4°C. As a negative control, 50 μl of a 1.5% red blood cell saline suspension was incubated with 50 μl of a mixture of 1 : 100 dilutions of UEA-1 and 100 mM of L-Fucose for 60 min at 4°C. These samples were washed two times (3,400 rpm/2 min), and resuspended in saline, and immunophenotypes were analyzed employing FACS Calibur™ (Becton Dickinson).

DNA extraction from saliva using magnetic bead method

Forty μl of a saliva sample and 100 μl of a solution containing 7 μl of resin and 93 μl of Lysis Buffer from the DNA IQ™ Kit (promega) were added to a 1.5 microcentrifuge tube and vortexed for 3 sec at high speed, and then incubated at room temperature for 5 min. The mixture was vortexed again for 3 sec once every min during the 5 min incubation, and placed the tube in the magnetic stand. All of the mixture was carefully removed and discarded without disturbing the resin pellet on the side of the tube, and then 100 μl of Lysis Buffer was added, the tube was removed from the magnetic stand, vortexed for 2 sec, and then placed again on the magnetic stand. All Lysis Buffer was carefully discarded without disturbing the resin. The resin was washed with 100 μl of 1× Wash Buffer, vortexed for 2 sec, and the tube was placed again on the magnetic stand. Wash Buffer was carefully discarded without disturbing the resin. The same washing procedure was repeated for a total of three washes. After the last wash, the tube was allowed to air-dry on the magmatic stand with the lid open for 5 min at room temperature, and 50-100 μl of Elution Buffer was then added and the lid was closed. The mixture was vortexed for 2 sec at high speed, and

incubated at 65°C for 5 min. The tube was removed from the heat, vortexed for 2 sec at high speed, and immediately placed on the magnetic stand. Finally, the DNA-containing solution was carefully transferred to another tube, and stored at 4°C or at -20°C.

ABO genotyping by polymerase chain reaction amplification of the specific allele (PASA)

The type of PASA reaction distinguishes the 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. The details of the procedure and validation of the PASA method for ABO genotyping were reported elsewhere (12-14). Briefly, for the six major ABO genotypes and ABO subtypes : *A/O*, *A/A*, *B/O*, *B/B*, *O/O*, *A/B*, and A_2B_3/O, three specific bands (379, 104, and 52 bp), two specific bands (379 and 52 bp), four specific bands (379, 224, 104, and 52 bp), two specific bands (224 and 52 bp), two specific bands (379 and 104 bp), three specific bands (379, 224, and 52 bp) and four specific bands (379, 104, 52, and 55 bp) were amplified, respectively. In this present study, we modified specific primer designs and PCR conditions from the conventional PASA method to improve the specificity and stability of allele-specific amplification with saliva samples. 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 the primer sequences and all possible specific bands of ABO genotype patterns obtained with the PASA method are given in Tables 1 and 2, respectively (12-14).

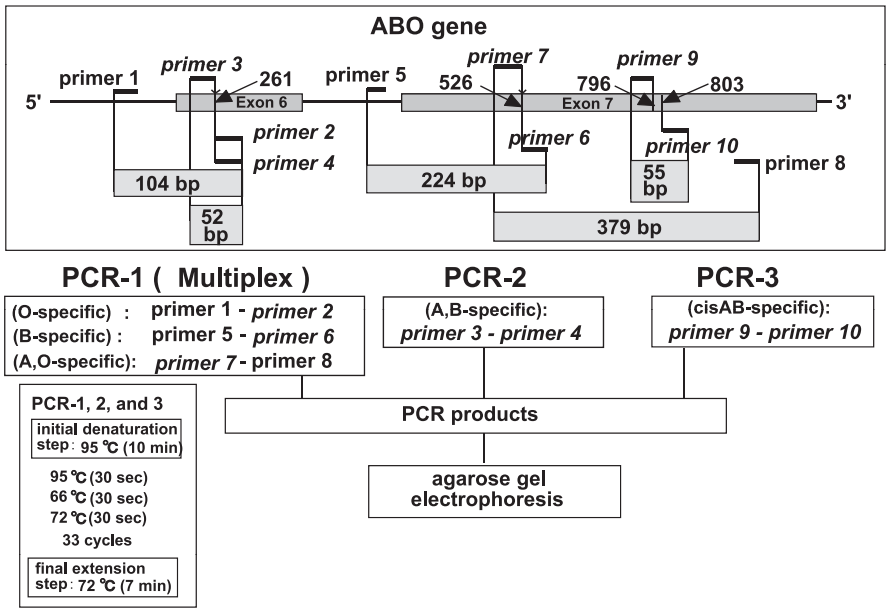

Figure 1 Scheme of amplification and analysis of specific ABO alleles using the PASA method.
The 3' endbase of primers 2, 3 and 4, 6, 7, and 9, and 10 corresponded to the nucleotide sequences of O, A and B, B, A, and O, and cisAB alleles, respectively. Primers 1, 5, and 8 corresponded to the nucleotide sequence of the ABO allele. Allele-specific DNA fragments of the O allele (104 bp), A and B alleles (52 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. Three (104, 224, and 379 bp) of five fragments were co-amplified in a single PCR 1 (multiplex-PCR). Primers 2, 6, and 10 were inserted at an artificial mismatched nucleotide at the second nucleotide from the 3' end of primers.

Table 1 Patterns of PASA method for all possible ABO genotypes

bp (Allele-specific band)	*A/O*	*A/A*	*B/O*	*B/B*	*O/O*	*A/B*	cisAB		
							A_2B_3/O	A_2B_3/A_1	A_2B_3/B
(a) 379 (A,O-specific)	+	+	+	-	+	+	+	+	+
(b) 224 (B-specific)	-	-	+	+	-	+	-	-	+
(c) 104 (O-specific)	+	-	+	-	+	-	+	-	-
(d) 52 (A,B-specific)	+	+	+	+	-	+	+	+	+
(e) 55 (cisAB-specific)	-	-	-	-	-	-	+	+	+

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

Table 2 The sequences of primers used in this study

Name	Sequence (5'→3')	Length (bp)
(1) fy-57	GAATTCATGTGGGTGGCACCCTGCCA	26
(2) 261-O2G	AGACAATGGGAGCCAGCCAAGGGGGA	26
(3) AB-261N	AGACAATGGGAGCCAGCCAAGGGGTC	26
(4) 261-AB	GAATTCAGGAAGGATGTCCTCGTGGTG	27
(5) fy-43	GGATCCAGGGGTGCACGGCCGGCGGC	26
(6) 526-B2	CTGCCAGCGCTTGTAGGCGTC	21
(7) 526-A	CAGCTGTCAGTGCTGGAGGTGC	22
(8) Fy-2	CCGTTGGCCTGGTCGACCATCATGGCCTG	29
(9) 796-A	AAGGACGAGGGCGATTTCTACTACC	25
(10) 803-B2	TCTTGCACCGACCCCCCGAAGAATG	25

The 3' endbase of primers (2), (3) and (4), (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 (1), (5), and (8) corresponded to the nucleotide sequence of the ABO allele. Primers (2), (6), and (10) were inserted at an artificial mismatched nucleotide at the second nucleotide from the 3' end of primers.

PCR conditions

PCR 1, 2, and 3 amplifications using the two sets of primers were carried out in a final volume of 25 µl, containing 10-20 ng of DNA, 50 pmol of each primer, 1 µl of 360 GC enhancer, and 12.5 µl of AmpliTaq Gold 360 Master Mix (Applied Biosystems). PCR 1, 2, and 3 amplification conditions were an initial denaturation step at 95°C for 10 minutes, followed by 33 cycles of denaturation at 95°C for 30 sec, annealing at 66°C for 30 sec, and extension at 72°C for 30 sec, and the last cycle extension was performed for 7 min at 72°C, followed by a quick chill to 4°C. The PCR product was mixed with gel of loading buffer and applied to 3% agrose gel (Wako, Osaka, Japan) that contained 0.5 µg/ml of ethidium bromide. Electrophoresis was visualized with Printgraph (ATTO, Tokyo, Japan).

RESULTS

Expression of A, B, and H antigens on red blood cells

The expression of A, B, and H antigens on red blood cells of various phenotypes was quantitatively compared by fluorescence labeling. Figure 2 summarizes the distribution patterns with respect to

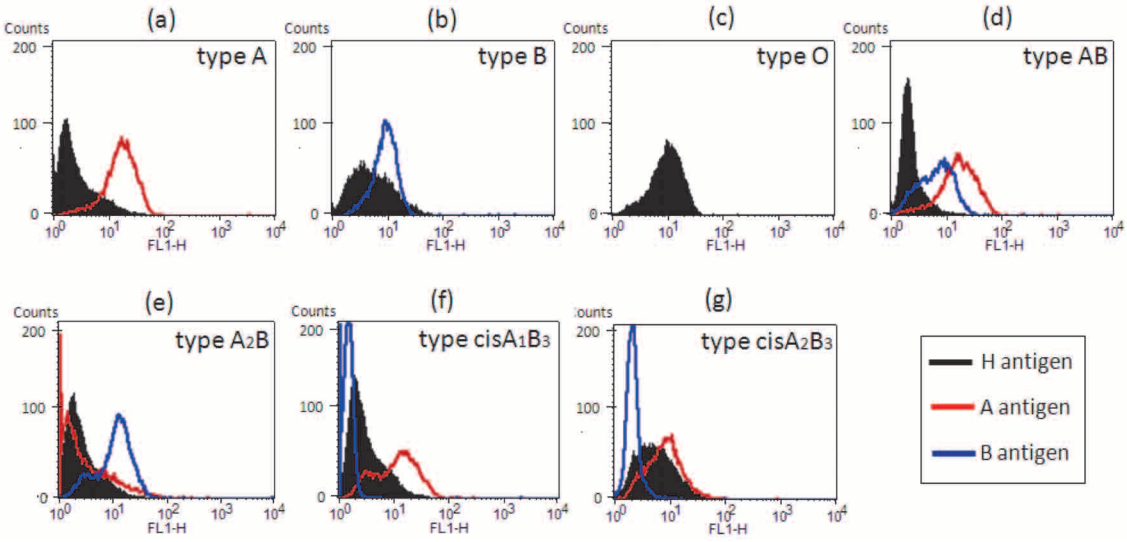

Figure 2 The expression patterns of A, B, and H antigens of red blood cells of four principal ABO blood types and three subtypes of type AB by flow cytometry.
(a), type A ; (b), type B ; (c), type O ; (d), type AB, (e), type A_2B, (f), type $cisA_1B_3$; (g), type A_2B_3. The horizontal axis shows fluorescence intensity and the vertical axis shows cell counts for each antigen. Solid red and solid blue line histograms show A and B antigens on red blood cells, respectively, and the solid black histogram shows H antigens on red blood cells.

the intensity of the fluorescence of red blood cells of various representative phenotypes labeled for A, B, and H antigens.

When red blood cells with the various phenotypes were labeled with anti-A and/or anti-B antibodies and UEA-1 lectin, A, B, O, AB, A_2B, cisA_1B_3, and cisA_2B_3 could be clearly distinguished. Variations in fluorescence patterns could be used to compare differences between expressions of A and/or B antigens, and H antigens on red blood cells with various phenotypes. Figure 3 shows the percentage of A, B, and H antigens on red blood cells of types A, B, O, AB, A_2B, cisA_1B_3, and cisA_2B_3. For type A, the positive rate of A antigen was 98.0 ± 0.9%, and was clearly higher than type A_2B (49.1%) and cisA_2B_3 (72.9 ± 6.5%), and slightly higher than type AB (94.0 ± 1.6%) and cisA_1B_3 (94.5 ± 0.8%). For type B, the positive rate of B antigen was 97.5 ± 1.0%, and was clearly higher than type cisA_1B_3 (10.8 ± 0.9%) and cisA_2B_3 (6.9 ± 4.0%), and higher than type AB (84.9 ± 4.2%), and slightly lower than A_2B (98.8%). For type O, the positive rate of H antigen was 76.6 ± 13.5%, and was reduced in the order of type B (27.8 ± 7.0%), A (18.8 ± 4.1%), and AB (9.7 ± 4.0%). On the other hand, three subtypes of type AB were clearly higher than type AB, and the positive rate of H antigen was high in the order of type A_2B (40.5%), cisA_1B_3 (47.3 ± 2.1%), and cisA_2B_3 (62.7 ± 7.9%). These data show a reciprocal relationship between the expression of A and/or B antigens, and H antigens on red blood cells in subtypes of type AB.

ABO genotyping by PASA

In this study, the type of PASA reaction could be used to distinguish the different ABO genotypes of genomic DNA extracted from the saliva and/or blood samples donated from volunteers, and 6 members belonging to one cisA_2B_3 pedigree on the basis of the molecular size of allele-specific amplification products that contain 261, 526, 796, and 803 nucleotides (the sites of amino substitution) in ABO allelic cDNA.

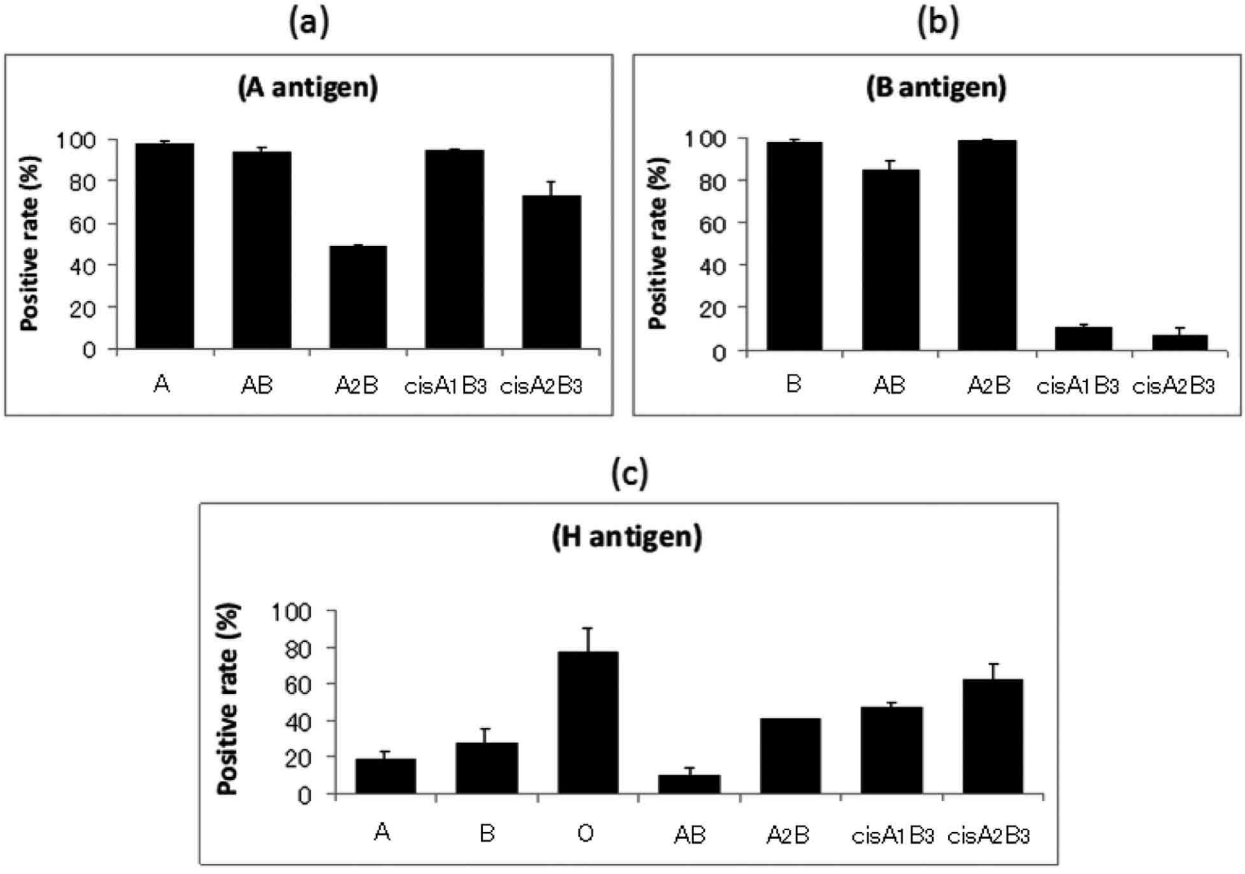

Figure 3 Quantitative comparison of A and/or B and H antigens of red blood cells of A, B, O, AB, A_2B, cisA_1B_3, and cisA_2B_3 analyzed by flow cytometry.
(a), The positive rate of A antigen (mean ± SD) ; (b), The positive rate of B antigen (mean ± SD) ; (c), The positive rate of H antigen (mean ± SD), A, B, O, and AB (n=5), A_2B (n=1), cisA_1B_3 (n=2), and cisA_2B_3 (n=5).

As shown in Figure 4, all genes of the five major ABO genotypes, *A/O*, *A/A*, *B/O*, *O/O*, and *A/B*, except for *B/B*, were amplified using DNA extracted from saliva ; three specific bands (379, 104, and 52 bp) for *A/O*, two specific bands (379 and 52 bp) for *A/A*, four specific bands (379, 224, 104, and 52 bp) for *B/O*, two specific bands (379 and 104 bp) for *O/O*, and three specific bands (379, 224, and 52 bp) for *A/B*. Under this PASA condition, only the specific bands were amplified in each genotype (band of 560 bp in PCR 1 is a common band in all blood types). In addition, Figure 5 shows a comparison of analysis with DNA extracted from saliva and blood obtained from the same person. The analysis of DNA extracted from blood employed a previously established method (12-14). The results of analysis using saliva were the same as those using blood, and could be used to distinguish the ABO genotypes.

Figure 6 shows patterns of amplification of typical types of *A/O*, *O/O*, and *cisA₂B₃/O* and Figure 7 summarizes the phenotype and genotype of the

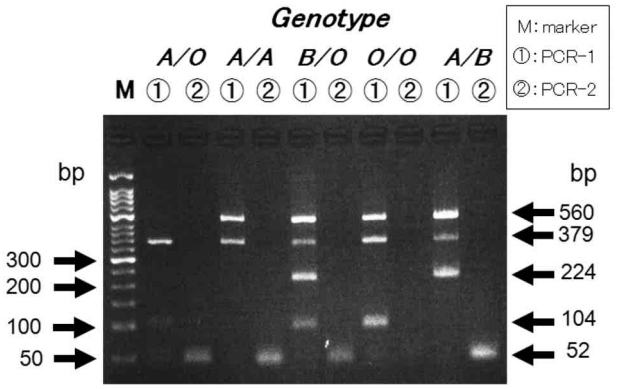

Figure 4 Electrophoretic patterns of PCR products in the 5 major ABO genotypes (*A/O*, *A/A*, *B/O*, *O/O*, and *A/B*).
Genomic DNA extracted from saliva was amplified employing the PASA method using 4 primer sets [①, PCR 1(primers 1 and 2, primers 5 and 6, and primers 7 and 8), ②, PCR 2 (primers 3 and 4)]. M, 50 bp DNA size marker, Band of 560 bp in PCR 1 is a common band in all types.

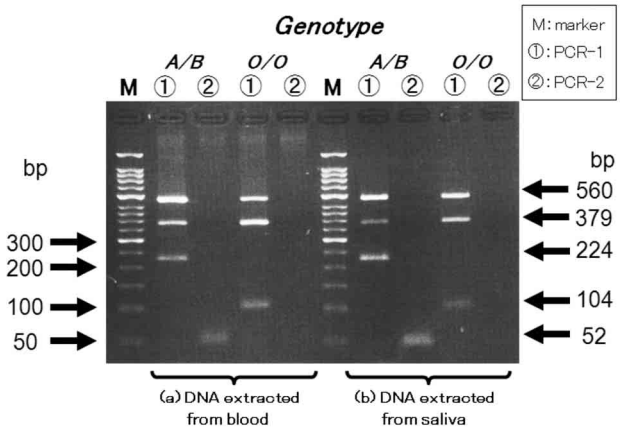

Figure 5 Electrophoretic patterns of PCR products in AB and O genotypes.
DNA extracted from saliva (a) and blood (b) obtained from same person was amplified with the PASA method using 4 primer sets [①, PCR 1(primers 1 and 2, primers 5 and 6, and primers 7 and 8), ②, PCR 2 (primers 3 and 4)]. M, 50 bp DNA size marker, Band of 560 bp in PCR 1 is a common band in all types.

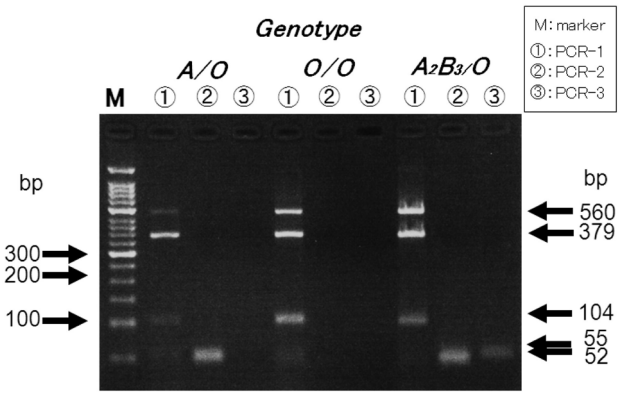

Figure 6 Electrophoretic patterns of PCR products in typical type *A/O*, *O/O*, and *cisA₂B₃/O*.
DNA extracted from saliva was amplified employing the PASA method using 5 primer sets [①, PCR 1(primers 1 and 2, primers 5 and 6, and primers 7 and 8), ②, PCR 2 (primers 3 and 4), ③, PCR 3 (primers 9 and 10]. M, 50 bp DNA size marker, Band of 560 bp in PCR 1 is a common band in all types.

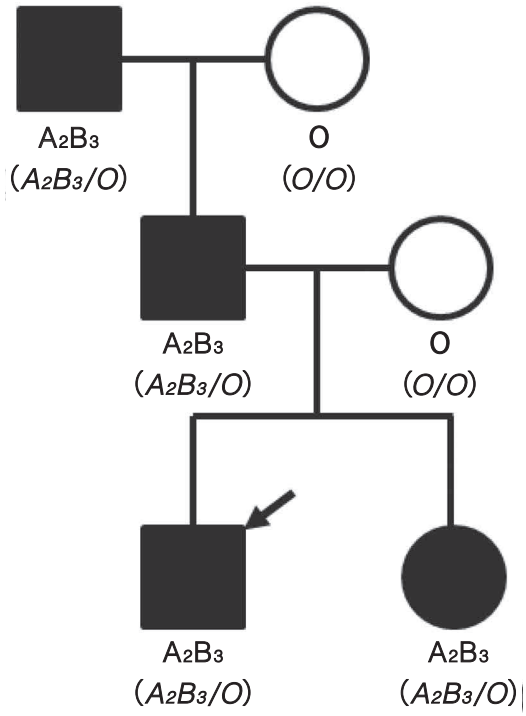

Figure 7 A pedigree chart of cisA_2B_3 family. Black : subjects with type cisA_2B_3, arrow : propositus.

cisA_2B_3 family pedigree. All genes of the six persons' ABO genotypes in the cisA_2B_3 pedigree were amplified using DNA extracted from saliva : 379, 104, 52, and 55 bp for the grandfather, 379 and 104 bp for the grandmother, 379, 104, 52, and 55 bp for the father, 379 and 104 bp for the mother, and 379, 104, 52, and 55 bp for the two children (data not shown except for the propositus and mother). Therefore, the grandfather, father, and two children were determined to be type cisA_2B_3, and the grandmother and mother were determined to be type O.

DISCUSSION

The ABO blood group system is the most important in both transfusion and organ transplantation, especially the evaluation of the adaptability of a donor's blood cells with bone marrow transplantation and hemocyte survival confirmation. Furthermore, the ABO blood group system is one of the most important markers of personal identification in forensics.

Currently, the ABO blood group antigens are determined by tube or column hemagglutination methods using anti-A or anti-B antibodies. These techniques are simple, and are sufficient for routine ABO blood group typing in transfusion, but such hemagglutinations are merely observations of a secondary reaction in antibody sensitization on the red blood cell membranes (18), and often show unreliable results in determining the blood type antigens, and it is difficult to quantify ABO antigens on red blood cells. Thus, we attempted to develop a quantitative analysis method for A, B, and H antigens on red cells by employing flow cytometric analysis. This analysis can observe the first phase of the binding state of antibodies (19), and can compare the number of antigens on the red blood cells by measuring the fluorescence intensity of binding antibody labeled with a fluorescent dye such as fluorescein isothiocyanate (20). Although this flow cytometric analysis is simple method, it is important to make examination in an optimal concentration of the first and second antibodies. Therefore, the optimal antibody concentration must be determined experimentally in each assay for stable results. In this study, we demonstrated that the positive rate of H antigen decreases in the order of type O, B, A, and AB. This result is consistent with that of Bianco *et al.* (21).

In addition, we were able to capture differences with a very small amount of A and B antigens on red blood cells, whereby the positive rate of A or B antigens of type AB, which has both A and B antigens, was lower than type A or B, which has either an A or B antigen. On the other hand, three subtypes of type AB were observed with a decrease of A and/or B antigens and an increase of H antigen. The amount of A antigen in cisA_2B_3 and A_2B was less than in types A and AB, and the amount of B antigen in cisA_2B_3 and cisA_1B_3 was less than in types B and AB. In contrast, the amount of H antigen was higher in all three subtypes. These results are consistent with those of Heier *et al.* (22) and Yazer *et al.* (23). Therefore, these subtypes of type AB are distinguished by decreased amounts of A and B antigens and an increased amount of H antigen on red blood cells. Detecting these subtypes is important, especially in blood transfusion and resolving paternity using the ABO blood group system.

In recent years, the sequences of ABO blood group genes in chromosome 9q34 have been determined, and it has become possible to detect ABO genotyping using molecular biology techniques (9-16). We previously reported that the PASA method is useful for detecting the ABO genotype with DNA extracted from the blood. However, blood sampling is painful and, in same cases, blood can not be drawn. Then, we attempted to establish an analysis method using DNA extracted from saliva, which can be collected non invasively. The collection of saliva is very easy, but the amount of DNA extracted from saliva is trace, and it was difficult to analyze ABO genotyping by employing a previously established PASA method. Therefore, it was necessary to improve the DNA extraction and PASA methods. We succeeded in extracting DNA of a sufficient quality and quantity from saliva using the magnetic beads method with the DNA IQ™ Kit. On the other hand, the PASA method was modified by altering the PCR conditions and primer design. Originally, the PASA method was developed for allelic analysis of mutation. Therefore, the design of the primer is very important, but it is not easy to design (24-25). This method is based on the fact that PCR amplification occurs only when the 3' endbase of the primer is matched to nucleotide No.261, 526, 796, or 803 of the ABO allelic cDNA, and three of five regions of allelic DNA were co-amplified in a single PCR (multiplex-PCR) in this study. Then, to improve the specificity of allele-specific amplification, we designed allele-specific primers in which a single artificial mismatched nucleotide was inserted at the

second nucleotide from the 3' end of primers (26), and determined 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, and amplified band patterns were consistent with the analysis results using DNA extracted from the blood.

In particular, this PASA method is useful for analyzing the type cisAB (i.e., cisA_1B_3, cisA_2B_3, and cisA_2B) family. Types cisA_1B_3, cisA_2B_3, and cisA_2B are very rare phenotypes, and have genotypes of A_2B_3/A_1, A_2B_3/O, and A_2B_3/B, respectively, and the A_2B_3 gene is a special gene in which A and B genes are located on the same chromosome. Types A_2B_3 and A_1B_3 are easily distinguished from the normal type AB due to the presence of anti-B and anti-A antibodies, but it is difficult to discriminate between cisAB (A_2B_3/A_1, A_2B_3/O, *or* A_2B_3/B) and transAB (A_1/B_3, A_2/B, *or* A_2/B) by serological tests alone. Ultimately, a pedigree study is required to determine type cisAB. On the other hand, the PASA method can determine type cisA_2B_3 quickly and easily with DNA only extracted from saliva.

In conclusion, we developed two analysis methods based on flow cytometric and PASA analyses for ABO phenotyping and ABO genotyping. Flow cytometric analysis can quantify A, B, and H antigens on red blood cells, and the PASA method allows the simple and rapid detection of multiple single nucleotide polymorphism (SNP) sites on the ABO gene. These methods are simple and useful for judging the ABO blood group system and genotyping, and are 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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