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Natsuko Mizuno,[1] D.V.M.; Takeshi Ohmori,[1] Ph.D.; Kazumasa Sekiguchi,[1] M.Sc.; Toru Kato,[2] B.Sc.; Toshinori Fujii,[3] B.Sc.; Koji Fujii,[1] Ph.D.; Tomoko Shiraishi,[1] M.Sc.; Kentaro Kasai,[1] Ph.D.; and Hajime Sato,[1] D.V.M., Ph.D.

Alleles Responsible for ABO Phenotype-Genotype Discrepancy and Alleles in Individuals with a Weak Expression of A or B Antigens*

ABSTRACT: ABO types obtained from evidentiary samples have been used effectively to obtain the initial information leading to the apprehension of culprits in Japanese criminal investigations. A simple ABO genotyping method using multiplex sequence-specific PCR and capillary electrophoresis was developed as a supplement to serological ABO typing. Limitations in predicting a phenotype based on genotype were evaluated using 1134 randomly selected Japanese peripheral blood samples. A concordance rate of 99.82% (1132/1134 samples) was found between genotypes and phenotypes defined as Groups A, B, AB, and O. Sequencing analysis revealed that one discrepant sample contained an O allele having a previously unreported point mutation at the primer binding site in exon 6, and another discrepant sample contained an O allele lacking the guanine deletion at nt 261 (the O301 allele). Therefore, the existence of such alleles must be given some consideration when predicting phenotype based on genotype.

KEYWORDS: forensic science, DNA typing, ABO, genotype-phenotype discrepancy, capillary electrophoresis, sequence-specific polymerase chain reaction, A_2 phenotype, Bm phenotype

ABO blood typing has been used effectively in Japanese criminal investigations for either including or excluding suspects, since ABO types obtained from evidentiary samples are usually one of the initial pieces of information available that could lead to the identification of a culprit. ABO types also have been used as one of the initial pieces of information when identifying the remains of missing persons and victims of crimes and disasters. Although ABO typing does not have a power of discrimination as high as VNTR or STR typing, it still has a great advantage in situations where reference biological samples are not available, since ABO types can be identified from sources such as medical records, blood donor cards, maternity health record books, and so on. Therefore, the demand for ABO typing from biological samples by criminal investigators has remained constant, although in 1992 DNA typing was introduced into Japanese criminal casework.

However, some difficulties exist in serologically typing bloodstain samples derived from certain weak subgroup phenotype individuals as well as body fluid samples derived from non- or weak secretors (1). Moreover, ABO types of weak A or B subgroup phenotype individuals are often simply recorded as A or B, respectively, in ABO records. In such cases, difficulties in detecting relevant antigens from bloodstains cannot be known from ABO records. This would lead to false exclusions due to superficial discrepancies between the ABO type in a record and the ABO type obtained from a bloodstain. Therefore, Japanese forensic biologists consider that ABO genotyping by DNA analysis should be used as a supplement to serological ABO typing in the field of forensic science.

Most ABO genotyping reported to date is based on PCR-RFLP (2–7), single-stranded conformational polymorphism (SSCP) (8–11) or sequence-specific PCR (12–17). PCR-RFLP analysis requires restriction enzyme digestions following PCR(s). This is laborious and time-consuming when performed routinely. Although SSCP analysis is used widely in research procedures, it would be impossible to perform it routinely due to the complexity of the electrophoresis involved. On the other hand, ABO genotyping using multiplex sequence-specific PCR would require only a single PCR reaction, followed by the separation of PCR products by ordinary denaturing gel electrophoresis. Therefore, we have developed a simple ABO genotyping method using multiplex sequence-specific PCR and capillary electrophoresis. The objective of this study was to validate the method by comparing ABO genotypes to phenotypes using 1134 Japanese blood samples and to analyze alleles that are responsible for a genotype-phenotype discrepancy and alleles in individuals with a weak expression of A or B antigens.

Materials and Methods

Samples and DNA Extraction

Blood was collected in EDTA from 1134 unrelated healthy Japanese subjects. DNA was isolated from 200-μL blood by MagNA Pure LC (Roche Diagnostics, Mannheim, Germany). The extraction procedure is based on the adsorption of the DNA to magnetic glass particles (18). After several washing steps to remove the cellular debris, the purified DNA was eluted in 10 m*M* TE

[1] National Research Institute of Police Science, 6-3-1, Kashiwanoha Kashiwa, Chiba 277-0082, Japan. Present address: Forensic Science Laboratory, Ishikawa Prefectural Police Headquarters, Kanazawa, Japan.
[2] Forensic Science Laboratory, Fukushima Prefectural Police Headquarters, Fukushima, Japan.
[3] Forensic Science Laboratory, Wakayama Prefectural Police Headquarters, Wakayama, Japan.

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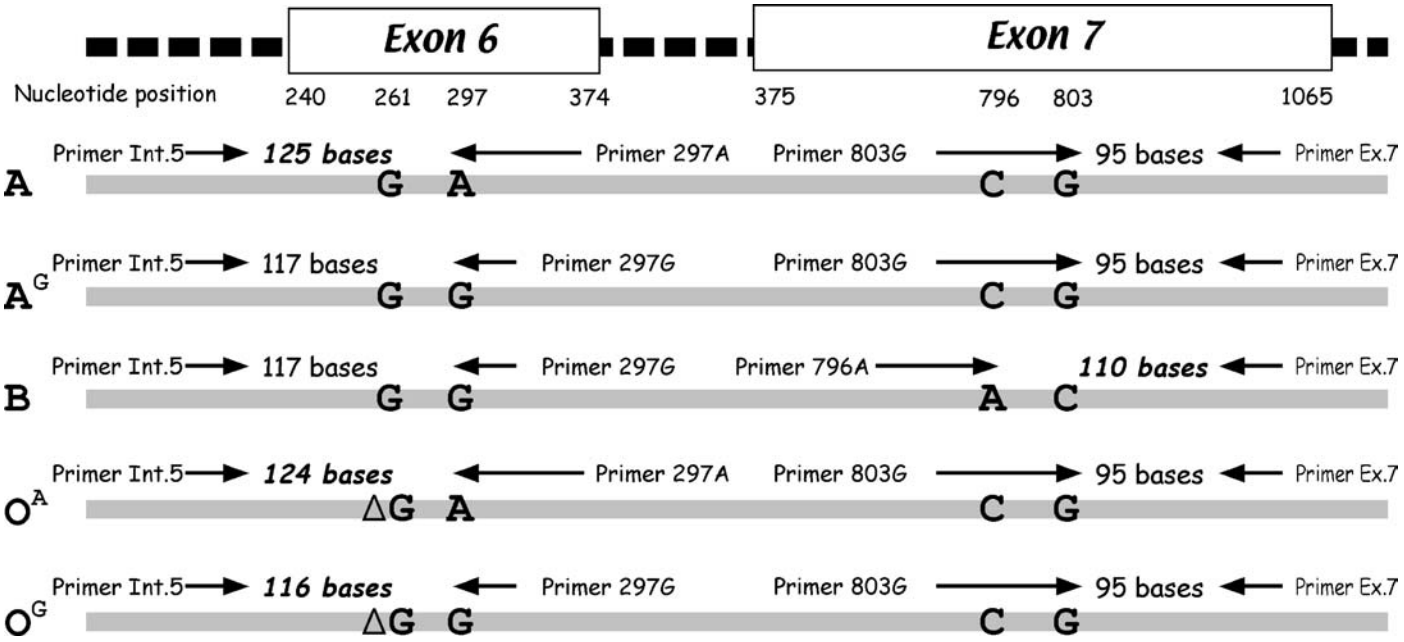

FIG. 1—*Grouped ABO alleles, sequence-specific primer positions, and amplified fragment lengths. The nucleotide positions indicated are relative to the A101 allele. Fragment lengths specific for only one allele are bold italicized.*

buffer (pH 8.0, 1 m*M* EDTA). DNA was quantified by a DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA).

Phenotyping

Monoclonal anti-A and anti-B sera (Biotest AG, Dreieich, Germany), anti-A_1 lectin (Wako Junyaku, Osaka, Japan), monoclonal anti-AB sera (Biotest AG, Dreieich, Germany), and anti-H lectin (Honen, Tokyo, Japan) were used for forward ABO phenotyping. Reverse ABO phenotyping was performed using sample plasma and known A_1, B, and O erythrocytes. When required for subgroup diagnosis, a saliva inhibition test was performed using rabbit polyclonal anti-A and anti-B sera (Wako Junyaku, Osaka, Japan), and anti-H lectin (Honen, Tokyo, Japan) at a titration value of 8.

Amplification and Typing

Multiplex sequence-specific PCR was employed for ABO genotyping. Various ABO alleles have been reported in the Japanese population (8–10,19,20). The major alleles can be grouped as A, A^G, B, O^A, and O^G based on four single nucleotide polymorphisms (SNPs) at nt 261, 297, 796, and 803 (Table 1) (5,8–10,15,17,19–22). Alleles are referred to by their serologic activity, and the superscripts A and G indicate that the allele has an A and G at nt 297, respectively. Six primers, three of which were labeled with the fluorescent dye NED (Applied Biosystems, Foster, CA), were used for multiplex sequence-specific PCR. The primer sequences are shown in Table 2. The ABO alleles, oligonucleotide primer positions relative to the consensus sequence (the A101 allele) (8), and amplified fragment lengths are illustrated in Fig. 1. The length includes a nucleotide added at the 3-' end of a fragment nontemplatedly (23). The O^A and O^G alleles have a point deletion of a G at nt 261 in exon 6 of the ABO gene, which results in frame shifting. One fragment from exon 6 and one from exon 7 were amplified from each allele. Two to four fluorescent-tagged fragments of different lengths were

TABLE 1—*Grouping of major ABO alleles in the Japanese population.*

Grouped Allele*	Major Alleles†	Phenotype
A	A102	A_1
	A101	A_1
A^G	A104	A_1
	A204	A_1 (heterozygotes with O), A_2(heterozygotes with B)
B	B101	B
O^A	O101	O
O^G	O201	O

* Grouped based on four SNPs at nt 261, 297, 796, and 803.
† Alleles are named according to the guidelines for human gene nomenclature.

TABLE 2—*Allele-specific primers used for ABO genotyping.*

Primer	Sequence	Position	Reference
Int.5	gtttCAGCTCCATGTGACCGCACGC	Intron 5	
297A	NED-agatacaGcGGATGTCGATGTTGAAT	313–297	(15)
297G	NED-AGGATGTCGATGTTGAAC	314–297	(15)
803G	gTCTACTACCTGGGGGGGT	788–805	
796A	gactgcAGGGCGATTTCTACTACA	779–796	(15)
Ex.7	NED-cGGCCTGGTCGACCATC	879–864	(15)

NOTE: Allele-differentiating nucleotides are underlined. Nucleotides not complementary to genomic DNA are in lower case. Nucleotides complementary to intronic sequences are in italics. Nucleotide positions indicated are relative to the A101 allele.

generated according to ABO genotypes. Since there is no fragment specific for only the A^G allele, the primer 803G is essential for differentiating the A^GB genotype (generated fragments; 95, 110, and 117 bases) from the BB genotype (generated fragments; 110 and 117 bases).

Multiplex sequence-specific PCR reactions were performed in a total volume of 10 μL containing 2 pmol primers int. 5, 297G and 796A, 4 pmol primers 297A and Ex 7, 8 pmol primer 803G, 0.5 to 2 ng of genomic DNA (up to 2 μL of a DNA sample in TE buffer), 1 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), and 1 X GoldST*R Buffer (Promega, Madison, WI). The concentrations of primers used were optimized so as to obtain a similar PCR yield for all the sequence-specific primers. Thermal cycling was performed in a GeneAmp® PCR system 9700 (Applied Biosystems): an initial denaturation step of 11 min at 95°C, 28 cycles consisting of 1 min at 94°C, 1 min at 54°C and 1min at 72°C, then a final extension step of 30 min at 60°C. The dye-labeled amplified products were resolved on an ABI Prism® 310 Genetic Analyzer (Applied Biosystems) following the manufacturer's recommendations. Data were collected by ABI Prism® Collection Software (Version 2.1) (Applied Biosystems) and were then analyzed by GeneScan® Analysis Software (Version 3.1) (Applied Biosystems). An ABO allelic ladder was prepared using samples whose genotypes (AO^G and BO^A) were determined by direct sequencing. A ±0.3-bp window around the size obtained from each fragment in the ABO allelic ladder was employed for genotyping using Genotyper® Software (Version 2.5) (Applied Biosystems) with the minimum peak height threshold for detection ≥150 relative fluorescence units (RFU).

Direct Sequencing

One fragment containing exon 6 and three overlapping fragments that cover exon 7 were amplified separately using the four primer pairs 1 to 4 (Table 3). PCR reactions were performed in a total volume of 10 μL containing 10 pmol of each primer, 10 ng of genomic DNA, 1 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), and 1 X GoldST*R Buffer (Promega, Madison, WI). Thermal cycling was performed using a GeneAmp® PCR system 9700: an initial denaturation step of 11 min at 95°C, 32 cycles consisting of 1 min at 94°C, 1 min at 60°C and 1min at 72°C for the amplification using Primer Pair 1, 32 cycles consisting of 1 min at 94°C, 2 min at 69°C, and 2 min at 72°C for the amplification using Primer Pair 2 to 4, then a final extension step of 10 min at 72°C. The ABI Prism® Big Dye terminators Version 2 cycle sequencing kit and an ABI Prism® 310 Genetic Analyzer (Applied Biosystems) were used for direct DNA sequencing according to the manufacturer's instructions. Data were analyzed using Sequencing Analysis Software™(Version 3.3) and Sequence Navigator Software (Version 1.0.1) (Applied Biosystems). When previously unreported mutations or an O allele lacking the guanine deletion at nt 261 were detected, we amplified a 2-kb DNA segment encompassing exon 6, intron 6, and exon 7 using the primer pair 261G or 261ΔG. Since the forward primers for these pairs discriminate the nucleotide at 261, an allele of interest was amplified selectively from a sample heterozygous for nt 261. The PCR conditions for 261G and 261ΔG were the same as Primer Pair 1 and Primer Pair 2 to 4, respectively, with the exception of changing the extension time in the PCR cycle to 8 min. Direct sequencing of the PCR product corresponding only to an allele of interest was then performed using Primer Pairs 1 to 4 with replacing 1fwd and 4rev by ABO-1F (or ABO-2F) and 7R, respectively.

Results

Only one discrepancy was observed in the ABO forward and reverse phenotyping in the 1134 Japanese blood samples. This sample was determined to be Type O in forward phenotyping, while it was determined to be B in reverse phenotyping, suggesting a Bm phenotype. A saliva inhibition test was performed to obtain a definite subgroup diagnosis. However, neither B nor H antigens were detected, probably due to the nonsecretor status of this individual, and thus it was classified as inconclusive. This sample was typed as Bm based only on the results obtained from forward and reverse phenotyping. Four weak A subgroup samples (one A_2 and three A_2B phenotype samples) were also found.

ABO genotyping using multiplex sequence-specific PCR and capillary electrophoresis were conducted with a negative control and positive controls consisting of known homozygotes (AA, BB) and heterozygotes (AB). The genotypes could be determined from

TABLE 3—*Primers used for direct sequencing.*

Primer Pair	Primer	Sequence	Position	PCR Product Size (bp)	Reference
1	1fwd	GTGCCAGAGGCGCATGTGGG	Intron 5	248* (247)*	
	1rev	TCGCCACTGCCTGGGTCTCTACC	Intron 6		
2	2fwd	attCCCCCGTCCGCCTGCCTTGC	Intron 6	366	
	2rev	attCGTAGAAGCCGGGGTGCAGGGTG	712–690		
3	3fwd	AGCGAGGTGGATTACCTGGTGTGCG	604–628	254	
	3rev	TGGTGGCAGGCCCTGGTGAGC	857–837		
4	4fwd	GGGTTCTTCGGGGGGTCGGTGCAAG	802–826	331	
	4rev	CTGCTAAAACCAAGGGCGGGAGGGGGA	3′-untranslated region		
261G†	ABO-1F	tattgtctacaGGATGTCCTCGTGGTG	246–261	2 k	(21)
	ABO-7R	TGGCAGCCGCTCACGGGTT	3′-untranslated region		
261Δ G‡	ABO-2F	gCCCTCGTGGTACCCCT	252–267	2 k	(21)
	ABO-7R	TGGCAGCCGCTCACGGGTT	3′-untranslated region		

NOTE: Nucleotides not complementary to genomic DNA are in lower case. Nucleotides complementary to intronic sequences are in italics. Allele-differentiating nucleotides are underlined. Nucleotide positions indicated are relative to the A101 allele. Primer 1fwd binding site is located at 5 ft. upstream of primer int.5 binding site on genomic DNA. Primer ABO-7R binding site is located at 5′ upstream of primer 4rev binding site on genomic DNA.

* The size of the PCR product is 248 bp for alleles having a guanine at nt 261, and 247 bp for alleles having a guanine deletion at nt 261.

† The primer pair 261G amplifies alleles having a guanine at nt 261.

‡ The primer pair 261Δ G amplifies alleles having a guanine deletion at nt 261.

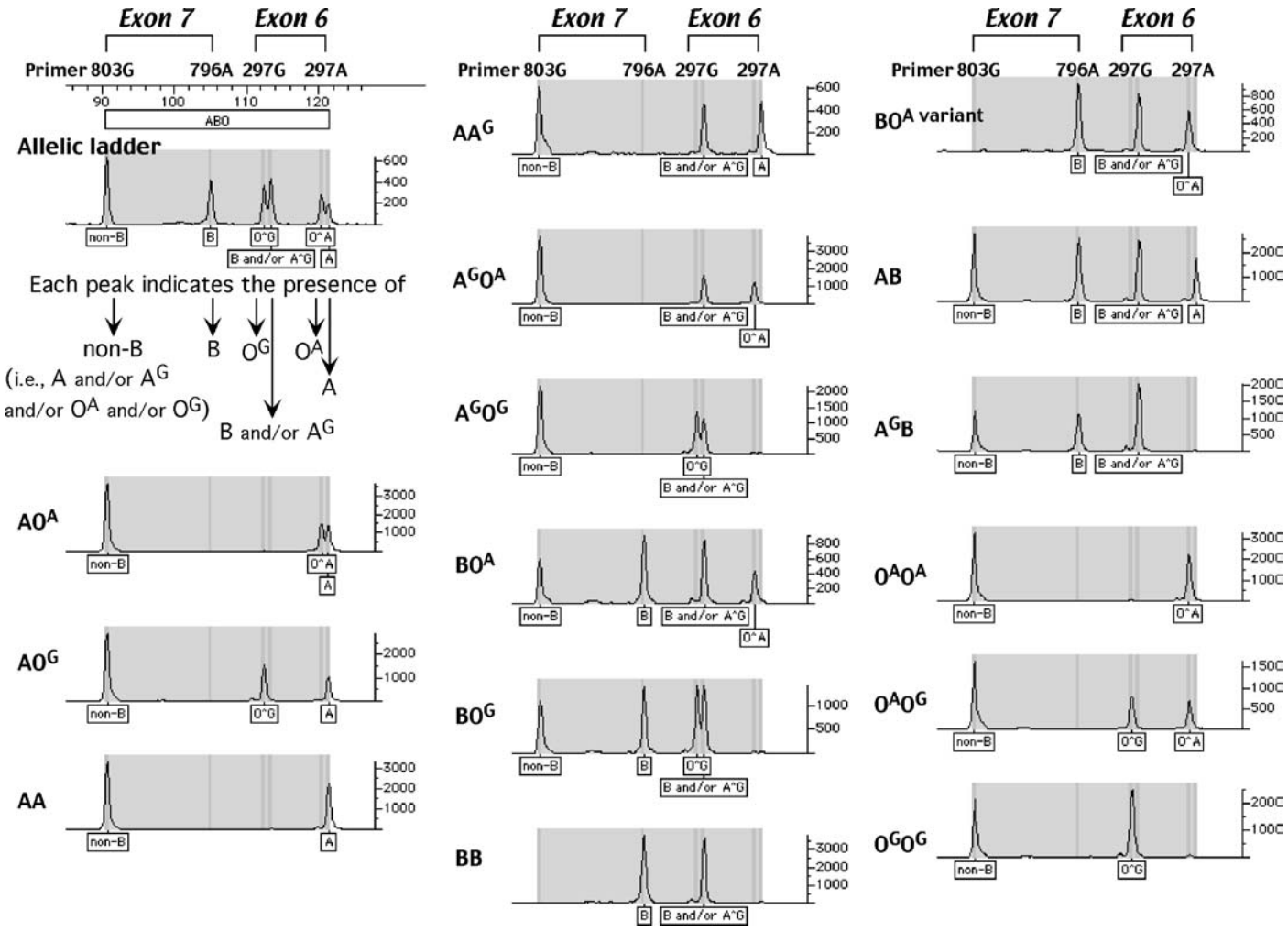

FIG. 2—*ABO genotyping using multiplex sequence-specific PCR and capillary electrophoresis. Electropherograms of all genotypes detected in 1134 Japanese subjects are shown (fragment size versus relative fluorescence units). A^G, O^A, and O^G are denoted as A^G, O^A, and O^G, respectively. Genotypes were determined unambiguously based onpeak patterns.*

all 1134 samples, although an unusual allele was detected in one sample (Fig. 2). This sample gave rise to a peak pattern that was inconsistent with any combination of known alleles. Namely, the fragments that were amplified from exon 6 were consistent with BO^A heterozygosity, but, the fragment amplified from exon 7 was consistent with BB homozygosity. The presence of the B allele and an unusual O^A allele ($O^{A\ variant}$) was indicated.

The results of ABO phenotyping and genotyping are shown in Table 4. The allelic frequencies for the ABO locus along with Hardy-Weinberg equilibrium results are shown in Table 5. ABO locus met Hardy-Weinberg equilibrium expectations in two analyses of a heterozygote deficiency and an exact test (24).

Of the 1134 samples, two samples exhibited a discrepancy between the genotype and phenotype as defined as Groups A, B, AB, and O (i.e., B phenotype but an A^GB genotype, and O phenotype but an AO^G genotype). A concordance rate of 99.82% was observed. To investigate the causes of these discrepancies, direct sequencing was performed using PCR products containing two alleles amplified from each heterozygous sample. Direct sequencing was also performed with the $BO^{A\ variant}$ genotype sample and with the subgroup phenotype samples (A_2, A_2B, and Bm). Sequencing data and the deduced most probable alleles present in each sample are shown in Table 6. The allele names in the deduced most probable alleles are given according to the guidelines for human gene nomenclature (8,19,20) except for the previously unreported alleles. One phenotype-genotype discrepant sample (B phenotype but A^GB genotype) contained a previously unreported point mutation from C to T at nt 306 in the primer 297A binding site. The other discrepant sample (O phenotype but AO^G genotype) contained a base substitution from C to T at nt 893, which is a characteristic base substitution of the O allele lacking the guanine deletion at nt 261 (the O301 allele) found in a Japanese subject (19). The $BO^{A\ variant}$ genotype sample contained a previously unreported point mutation from G to T at nt 801 in the primer 803G binding site. The A_2 phenotype sample contained a previously unreported point mutation from G to A at nt 778. The Bm phenotype sample displayed a sequence compatible with one containing the common B101 allele, which is responsible for the normal B phenotype and the O201 allele. All A_2B phenotype samples displayed a sequence compatible with containing the A204 allele (also referred to as the R101 allele) (10) and the B101 allele.

Further, using the Primer Pair 261G or 261ΔG, an allele of interest (i.e., potential O101(C306T), O301, O101(G801T), A102(G778A), and B101 alleles) was amplified selectively from each sample that was heterozygous for nt 261 and then sequenced. As a result, the presence of the O301 allele was confirmed, the three

TABLE 4—*Results of phenotyping and genotyping 1134 Japanese samples and the observed allele frequencies for the ABO locus.*

Phenotype		*n*	Frequency	Genotype	*n*	Frequency
A	A_1	458	0.404	AO^A	185	0.163
				AO^G	170	0.150
				AA	92	0.081
				AA^G	3	0.003
				A^GO^A	4	0.004
				A^GO^G	4	0.004
	A_2	1	0.001	AO^G	1	0.001
B	B	261	0.230	BO^A	122	0.108
				BO^G	110	0.097
				BB	27	0.024
				A^GB	***1***	***0.001***
				$BO^{A\ variant}$*	1	0.001
	Bm	1	0.001	BO^G	1	0.001
AB	A_1B	99	0.087	AB	97	0.086
				A^GB	2	0.002
	A_2B	3	0.003	A^GB	3	0.003
O	O	311	0.274	O^AO^A	88	0.078
				O^AO^G	158	0.139
				O^GO^G	64	0.056
				AO^G	***1***	***0.001***

NOTE: Samples in which discrepancy between phenotype and genotype was observed are bold italicized.

* The $O^{A\ variant}$ allele generated a PCR product corresponding to the O^A allele in exon 6, however, did not generate a PCR product corresponding to the O^A allele in exon 7.

TABLE 5—*Observed allele frequencies for the ABO locus in the Japanese population (n = 1134).*

Allele	*n*	Frequency
A	641	0.283
A^G	17	0.007
B	391	0.172
O^A	645	0.284
O^G	573	0.253
$O^{A\ variant}$*	1	0.0004
Observed homozygosity		23.90%
Expected homozygosity		25.40%
Heterozygote deficiency		0.925
Exact test		0.2693

* The $O^{A\ variant}$ allele generated a PCR product corresponding to the O^A allele in exon 6, however, did not generate a PCR product corresponding to the O^A allele in exon 7.

new alleles: O101(C306T), O101(G801T), and A102(G778A), were positively identified, and the presence of an allele having an identical DNA sequence to the B101 allele in exons 6 and 7 was confirmed in the Bm phenotype sample.

All AA and AA^G genotype samples were analyzed by direct sequencing using Primer Pair 4 to determine whether additional O301 alleles were present in these samples. None of the samples contained a base substitution at nt 893. Thus, only one O301 allele was identified out of 1134 Japanese subjects.

Discussion

A simple and reliable ABO genotyping system using sequence-specific PCR and capillary electrophoresis was developed. Based on four SNPs at nt 261, 297, 796, and 803, the five alleles, i.e., A, A^G, B, O^A, O^G, can be differentiated. The genotyping method was validated by comparing ABO genotyping to phenotyping using 1134 Japanese blood samples. A new $O^{A\ variant}$ allele and two phenotype-genotype discrepant cases were observed.

The $O^{A\ variant}$ allele contained a point mutation (from G to A at nt 801) at the primer 803G binding site in exon 7 compared to the O101 allele. Due to this mutation, a fragment indicating the presence of non-B-allele(s) (i.e., A and/or A^G and/or O^A and/or O^G) was not amplified from exon 7 of the allele. It is detected as the $O^{A\ variant}$ allele only when it is in heterozygotes with the B allele or in homozygotes. Otherwise (i.e., in heterozygotes with the O^A, O^G, A or A^G allele), the $O^{A\ variant}$ allele cannot be distinguished from the common O^A allele because exon 7 of the common non-B-allele (i.e., the O^A, O^G, A or A^G allele) generates the fragment indicating the presence of non-B-allele(s), thus resulting in a normal peak pattern in the electropherogram. The $O^{A\ variant}$ allele is not problematic since it would not lead to an incorrect phenotype prediction based on a genotype in combination with any allele.

The one discrepant sample (B phenotype and A^GB genotype) contained an allele with a point mutation at the primer binding site in exon 6, i.e., O101(C306T). Thus, missing the amplified fragment corresponding to the O^A allele in exon 6 was the cause of this discrepancy. In contrast to the $O^{A\ variant}$ allele, the O101(C306T) allele is very problematic, especially when it is in heterozygotes with the B allele, because this case leads to an incorrect phenotype prediction. Since PCR failure due to a mutation at a primer binding site is inevitable for any primer, the problem of this type is not limited only to the method described here.

The other discrepant sample (O phenotype and AO^G genotype) contained the previously reported O allele lacking the guanine deletion at nt 261 found in Japanese (i.e., the O301 allele) (19). The O301 allele has a base substitution at nt 893 from C to T compared to the A102 allele, which is the most common A allele in the Japanese population. Thus, the presence of the O allele lacking the guanine deletion at nt 261 (in other words, the presence of a nonfunctional A allele) was the cause of this discrepancy.

In both cases, the discrepancy was not caused by nonspecific PCR amplification, failure in the electrophoresis, or PCR failure that occurred in spite of the presence of an allele having a sequence that is perfectly complementary to the primers. In addition, all samples except the two samples mentioned above exhibited concordance between phenotypes and genotypes, and the frequencies of A, B, and O alleles observed in this study were similar with those calculated from 1,181,584 Japanese blood donors (25). This validates the reliability of the genotyping system described here.

Although Ogasawara et al. found the O301 allele in a blood sample of a serum anti-A antibody-deficient O phenotype individual selected by routine serological testing (19), we observed one O301 allele from a normal O phenotype individual (i.e., possessing serum anti-A antibodies with common agglutinability) out of the 1134 randomly selected Japanese subjects. We estimated the frequency of the O301 allele as 0.044% in the Japanese population and 0.16% among O phenotype individuals. Using PCR-SSCP analysis, Ogasawara et al. also identified one R102 allele and three R103 alleles that did not contain the guanine deletion at nt 261 from 1032 Group O phenotype Japanese individuals possessing serum anti-A antibodies with common agglutination (19). Those alleles were not observed in the 311 O group phenotype individuals examined in this study. Conversely, while we observed one O301 allele in the 311 group O phenotype individuals, Ogasawara et al. did not observe this allele in the 1032 group O phenotype individuals.

The O allele lacking the guanine deletion at nt 261 was first identified by Grunnet et al. in a Danish population (4). This allele (i.e., the O303 allele) has a characteristic base substitution from G

TABLE 6—*Direct sequencing of exon 6 and exon 7 amplicons obtained from samples in which a phenotype-genotype discrepancy was observed and ABO subgroup phenotype samples.*

Phenotype	Genotype by Sequence-Specific PCR	Nucleotide Position																	Probable Alleles	*n*
		Exon 6								Exon 7										
		261G†	297A†	306C†	467C†	526C†	646T†	657C†	681G†	703G†	771C†	778G†	796C†	801G†	803G†	829G†	893C†	930G†		
B	***A^{G}B***	G/Δ*	R	Y		S		Y		R			M		S			R	O101(C306T)‡/B101	1
O	***AO^{G}***	G/Δ*	R		Y		W		R		Y					R	Y		O301‡/O201	1
B	BO$^{A\ variant}$	G/Δ*	R			S		Y		R			M	K	S			R	B101/O101(G801T)‡	1
A_2	AO^{G}	G/Δ*	R		Y		W		R		Y	R				R			A102(G778A)‡/O201	1
Bm	BO^{G}	G/Δ*	G			S	W	Y	R	R	Y		M		S	R		R	B101‡/O201	1
A_2B	A^{G}B		G			G		T		A	Y		M		S	R		R	A204/B101	3

Samples in which a discrepancy between phenotype and genotype was observed are bold italicized. Only changes from the consensus sequence (A101) are shown. The nucleotide positions indicated are relative to the A101 allele. Allele names in the probable alleles were given according to the guidelines for human gene nomenclature. IUPAC-IUB codes are used (K: G or T, M: A or C, R: A or G, S: G or C, Y: C or T, W: A or T).

* At position 261, G/Δ indicates G or the deletion of a G.

† G, A, C and T following a nucleotide position indicates the nucleotide in the consensus sequence at that position.

‡ An allele of interest was amplified selectively from each heterozygous sample using the Primer Pair 261G or 261ΔG and then sequenced. The presence of the O301 allele was confirmed and three new alleles, i.e., O101(C306T), O101(G801T), and A102(G778A), were identified.

to A at nt 802, which results in an amino acid substitution of glycine to arginine at codon 268. The frequencies of the O303 allele were reported as 1 to 3% in various Caucasian populations (11,14–16,26,27). However, the O303 allele has not yet been identified in an Asian population. In this study, the O303 allele was not observed and only one O301 allele was observed. Thus, O alleles lacking the guanine deletion at nt 261 appear to be quite rare in the Japanese population compared to Caucasian populations, consistent with the report by Ogasawara et al. (19), though the observed alleles were different between their study and this study.

Most of the mutations found on A or B weak subgroup alleles were located in exon 7 (9,10,28–30). It is known that the molecular basis of blood groups can differ between populations (31). In Caucasians, the A105 allele is predominant among alleles being responsible for the A_2 phenotype and is characterized by a single C deletion in a series of the three C's at nt 1059 to 1061 (29). This single C deletion in a series of the three C's at nt 1059 to 1061 was not present in the Japanese A_2 phenotype individual observed in this study. Instead, the A102 allele with a novel point mutation from G to A at nt 778 is carried by this individual. The A102 allele is known to be responsible for the A_1 phenotype and is the most common A allele in the Japanese population (19). The mutation observed in the A_2 phenotype individual results in an amino acid substitution from glutamic acid to lysine at codon 260. Therefore, this mutation might be responsible for the A_2 phenotype.

All three A_2B phenotype samples were genotyped as A^GB. The A^G allele includes the A104 allele and the A204 allele (8,10). While the A104 allele is known to be responsible for the A_1 phenotype, the A204 allele is known to be responsible for the A_2 phenotype in heterozygotes with B alleles and for the A_1 phenotype in heterozygotes with O alleles (10). The chromatogram of the direct sequences of each A_2B phenotype sample exhibited a superposition of two sequences of the A204 and the B101 allele. Thereby, the A^G alleles in all the A_2B phenotype samples were most probably the A204 allele.

The Bm phenotype sample contained an allele that had the same sequence as the normal B allele (B101) in exons 6 and 7. This was not surprising, because it has been reported that there were B weak subgroup alleles that contained no differences from the normal B allele in exons 6 and 7 (30) and in the full coding region (exons 1–7) and regulatory regions (28). The cause of the weak B expression on erythrocytes has not been clarified in such cases. The Bm phenotype is known to be one of the most frequently observed weak subgroup phenotypes in the Japanese population (25). While B and H antigens can be detected in saliva derived from Bm phenotype individuals in a secretor status by an inhibition test, B antigens cannot be detected in a bloodstain by an absorption-elution test. By using ABO genotyping as a supplement to serological typing, an incorrect ABO grouping of Bm bloodstain could be prevented.

Since the production of human anti-A and anti-B sera have been discontinued, human polyclonal antisera are being replaced by mouse monoclonal antibodies. Determining ABO types of stain samples using mouse monoclonal antibodies empirically seems to require examiners to have more experience than when human polyclonal antisera is used, even when stain samples are derived from common phenotype individuals. Therefore, when both serological typing using mouse monoclonal antibodies and ABO genotyping are performed and a concordance between a phenotype and a genotype is observed, examiners can determine the ABO phenotype with an even higher level of confidence. On the other hand, when a phenotype-genotype discrepancy is observed, the result must be classified as inconclusive in a casework report because there are both possibilities that the phenotype obtained by the serological typing procedure reflects the true phenotype of the relevant individual, and a phenotype predicted based on the genotype reflects the true phenotype of the relevant individual.

A simple and reliable ABO genotyping system has been developed. A concordance rate of 99.82% was observed between genotypes obtained by the ABO genotyping method described here and phenotypes defined as Groups A, B, O, and AB. The major obstacles in predicting the correct ABO group based on the genotype were point mutations at the primer binding sites and O alleles lacking the guanine deletion at nt 261, both of which appeared to be quite rare in the Japanese population. If Japanese casework examiners are aware of the above facts, it is likely that the ABO genotyping assay described here can be used as a useful supplement to serological ABO typing in the analysis of evidentiary samples.

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Additional information and reprint requests:
Natsuko Mizuno, D.V.M., Chief
Forensic Science Laboratory, Ishikawa Prefectural Police Headquarters
1-1 Kuratsuki Kanazawa
Ishikawa 920-8553, Japan
E-mail: mizuno@nrips.go.jp