JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, January 2011, Vol. 56, No. S1
doi: 10.1111/j.1556-4029.2010.01630.x
Available online at: onlinelibrary.wiley.com

TECHNICAL NOTE

CRIMINALISTICS

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A Novel Method for ABO Genotyping Using a DNA Chip

ABSTRACT: ABO genotyping is often performed to identify the blood type of decomposed samples, which is difficult to be determined by a serological test. In this study, we developed a simple method for ABO genotyping using a DNA chip. In this method, polymerase chain reaction-amplified and fluorescent-labeled fragments in the ABO gene and primate-specific D17Z1 were hybridized with DNA probes on a chip designed to detect single nucleotide polymorphisms (SNPs) in the ABO gene and part of the D17Z1 sequence. Using blood samples from 42 volunteers and 10 animal species, we investigated whether the chip could be used to detect SNPs in the ABO gene and the D17Z1 sequence. This method was then applied to various forensic samples, and it was confirmed that this method was suitable for the simultaneous analyses of ABO genotyping and species identification. This method fulfills the recent need for the development of rapid and convenient methods for criminal investigations.

KEYWORDS: forensic science, ABO blood group system, ABO genotyping, single nucleotide polymorphisms, DNA chip, human identification

ABO blood typing has been used for many years as a general tool for the individual identification of biological forensic samples. Although most recent forensic laboratories rarely perform ABO typing for the individual identification of samples because of the development of the short tandem repeat typing technique, ABO blood type information is still important in some cases, such as the investigation into unknown cadavers and the screening of suspects. For ABO blood typing, the serological test has generally been performed; however, this method is difficult to apply to decomposed samples because of the instability of ABO antigens. Therefore, in many criminal investigation laboratories, ABO genotyping of DNA is often performed to identify the blood type of decomposed samples.

After identifying the structure of the ABO gene (1,2), many alleles of this gene were revealed to be responsible for the serological phenotypes (3). Comparing the major types of A, B, and O alleles, there is an O allele–specific single nucleotide deletion and B allele–specific nucleotide substitutions in exons 6 and 7 of this gene. In many existing ABO genotyping methods (4–9), the genotypes were determined by analyzing these single nucleotide polymorphisms (SNPs). Among these techniques, the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method (4) is most commonly used in criminal investigation laboratories. The PCR-RFLP method requires a restriction enzyme digestion step following the amplification of the ABO gene; however, this step is time consuming and can result in false ABO genotypes because of incomplete digestion (10).

On the other hand, it is well known that the use of DNA chips is a rapid and precise method for analyzing SNPs. We previously developed DNA chip methods that can detect SNPs in viral genomes to narrow down the geographical origins of unidentified cadavers (11,12). However, although Li et al. (13) reported a DNA chip method to analyze various SNP loci, including the ABO gene, there are few reports describing the use of DNA chip technology for ABO genotyping, despite the usefulness of this method for SNP typing. If a rapid and convenient ABO genotyping method is established using this technology, it should be useful not only for ABO genotyping of decomposed samples but also for various forensic cases, such as the screening of vast specimens before DNA typing. Moreover, using this method, many rare alleles of ABO gene in addition to the major alleles can be simultaneously analyzed because various types of DNA probe can be spotted on a same chip to detect each target sequence.

In this study, we developed a simple DNA chip as proof of concept, which can detect the major ABO allele–specific SNP sites. Furthermore, this chip was designed to detect the primate-specific DNA sequence D17Z1 (14–16), because homologs of the ABO gene are conserved in many species (17–22), and therefore there is a possibility that DNA samples derived from nonhuman species can be detected using this method. In the first step of this method, fragments of the ABO gene and D17Z1 were amplified using PCR and labeled with a fluorescent dye. Fluorescent-labeled amplicons were then hybridized with DNA probes on a chip

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Received 22 July 2009; and in revised form 28 Dec. 2009; accepted 9 Jan. 2010.

designed to detect SNP sites in the ABO gene and part of the D17Z1 sequence. The fluorescent signal on each probe was quantified by a detector. In this study, we verified whether the chip can be used to detect SNP sites of the ABO gene and the D17Z1 sequence and whether this method can be applied to various forensic samples.

Materials and Methods

Samples and DNA Extraction

All samples were obtained using procedures approved by the Institution Review Board of the National Research Institute of Police Science. Blood samples and hair from living individuals were obtained from Japanese volunteers. The aorta, hair, and blood of cadavers were obtained during medico-legal autopsies at Chiba University. Blood stains were made by spotting whole blood onto cotton cloth, and the samples were subsequently stored at room temperature in a dark room. DNA samples were extracted using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. ABO genotypes of all human DNA samples were determined by amplifying and sequencing fragments of the ABO gene.

DNA Chip Design

Seven SNP sites of the ABO gene (Table 1 and Fig. 1) were selected for ABO genotyping based on the sequences of five alleles (A101, A102, B101, O101, and O102), which are common in the Japanese population (7,23). To detect the O allele–specific single nucleotide deletion, a DNA probe set designed for the SNP site (261G/Δ; Δ, deletion of nucleotide) was fixed to a silicon chip (Toyo Kohan, Yamaguchi, Japan; [24]). The DNA probe set for the SNP site (930G/A) was also attached because this site was the most sensitive among the six B allele–specific nucleotide substitutions (Table 1) in a preliminary experiment (data not shown). For species identification, a DNA probe for the primate-specific genome sequence D17Z1 was also attached. The sequences of all DNA probes are shown in Table 2. Each DNA probe (10 μM) was spotted four times onto a chip with a diameter of 260 μm. The probe arrangement of the DNA chip is shown in Fig. 2*a*.

TABLE 1—*Single nucleotide polymorphism sites for major ABO alleles.*

	Nucleotide position						
Allele	261	526	657	703	796	803	930
A	G	C	C	G	C	G	G
B	G	G	T	A	A	C	A
O	Δ	C	C	G	C	G	G

Δ, deletion of nucleotide.

DNA Chip Analysis

The 528- and 786-bp fragments of the ABO gene (Fig. 1) and the 207-bp fragment of the D17Z1 region were amplified by multiplex PCR using three sets of primers, shown in Table 3. A 20-μL reaction mixture containing 1 μL of the template DNA, 0.5 μL of ABO 1-f, ABO 1-r, ABO 2-f, and ABO 2-r (10 μM each) primers, 0.5 μL of D17Z1-f and D17Z1-r (0.5 μM each) primers, 0.2 μL of Cy5-dCTP (GE Healthcare, Buckinghamshire, U.K.), 2 μL of dNTP mixture (0.5 mM each dNTP, 0.4 mM dCTP), 2 μL of 50% glycerol, 2 μL of Ex Taq buffer, and 0.1 μL of Ex Taq polymerase (Takara Bio, Shiga, Japan) was used for amplification. The DNA samples used in Fig. 3 were all extracted from 200 μL of whole blood and eluted in 100 μL of elution buffer with the DNA extraction kit. Therefore, DNA from 2 μL of whole blood (equivalent to around 50 ng of DNA) was used as a template. In the experiment shown in Table 4, we used 50 ng/μL DNA for each PCR. In Table 5, various concentrations (10–50 ng/μL) of DNA were used. We used 45 cycles (95°C for 20 sec, 58°C for 20 sec, and 72°C for 30 sec) for the amplification. A 2-μL aliquot of the reaction mixture was mixed with 1 μL of 3× SSC/0.3% SDS buffer and hybridized with the chip at 45°C for 60 min. The hybridized chip was washed with 1× SSC buffer and scanned with a fluorescent scanner, BIOSHOT (Toyo Kohan, Yamaguchi, Japan). A representative image is shown in Fig. 2*b*. We calculated the fluorescence ratio of each probe set, [261 (O)/261 (A, B)] and [930 (B)/930 (A, O)], from the average fluorescent intensity of each probe. As the fluorescent intensity of the 261 (O) probe should increase depending on the number of O alleles in each individual, the [261 (O)/261 (A, B)] ratio was expected to vary among the three genotype groups: OO, the genotypes heterozygous for the O allele (AO and BO, referred to in this paper as XO), and the other genotypes (AA, AB, and BB, referred to in this paper as XX). Similarly, the [930 (B)/930 (A, O)] fluorescence ratio was expected to vary among BB, the genotypes heterozygous for the B allele (AB and BO, referred to in this paper as BY), and the other genotypes (AA, AO, and OO, referred to in this paper as YY). Therefore, the ABO genotype of each sample could be determined from the fluorescence ratios of the two probe sets by setting threshold values between each genotype group.

TABLE 2—*Probe sequences.*

Probe	Sequence
261 (A, B)	5′-tcctcgtggtGacccctttgg-3′
261 (O)	5′-tcctcgtggtacccctttggc-3′
930 (A, O)	5′-acaagtacctGctgcgccac-3′
930 (B)	5′-acaagtacctActgcgccac-3′
D17Z1	5′-tctgtgtccttcgttcgaaacg-3′

Nucleotides corresponding to single nucleotide polymorphism sites are designated by capital letters.

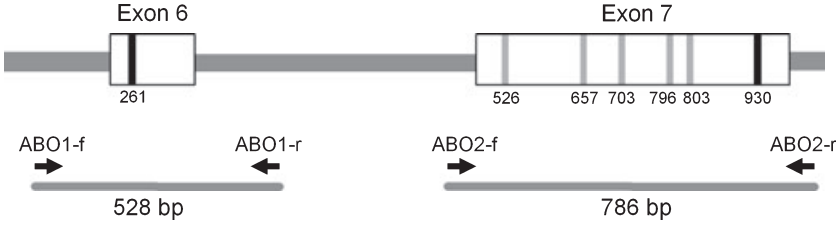

FIG. 1—*Single nucleotide polymorphism sites and amplified regions of the ABO gene. Primers used for the polymerase chain reaction amplification of the ABO gene are shown as arrows.*

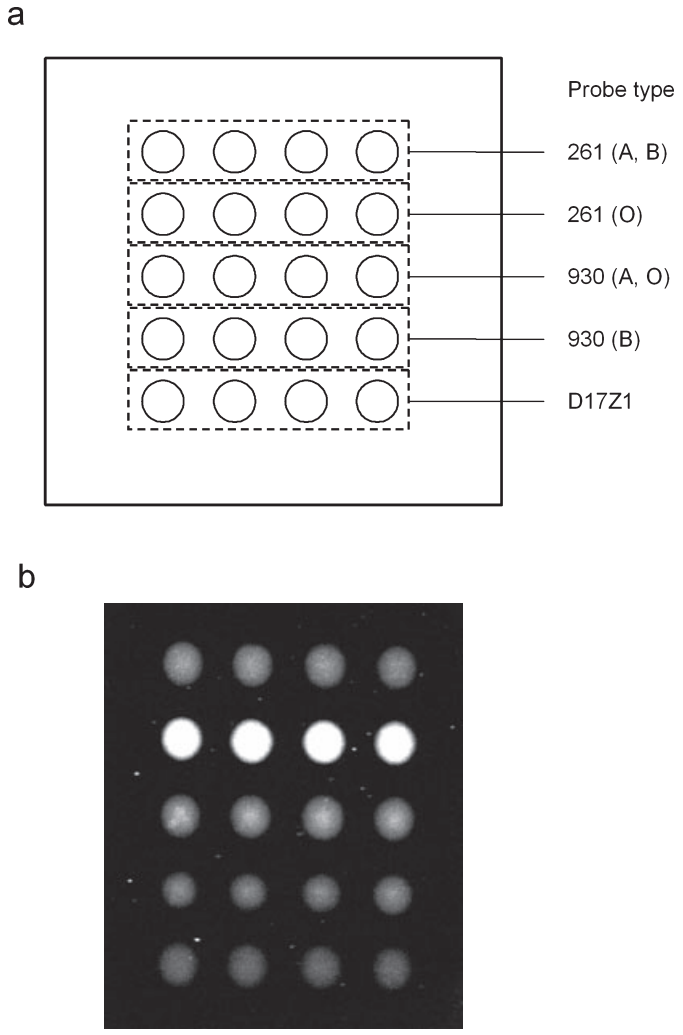

FIG. 2—*Probe arrangement and fluorescent image of the DNA chip. (a) Probe arrangement of the DNA chip. Each probe in Table 2 was spotted four times onto a chip. (b) Representative image of the DNA chip scanned by a fluorescence detector for an AO sample.*

Results and Discussion

Using blood samples from 42 volunteers, we first measured the fluorescence ratio values of the two probe sets, [261 (O)/261 (A, B)] and [930 (B)/930 (A, O)]. As shown in Fig. 3*a*, the average [261 (O)/261 (A, B)] ratio varied among the three genotype groups (OO, XO, and XX; n = 15, 17, and 10, respectively). Similarly, the average [930 (B)/930 (A, O)] ratio varied among the three genotype groups (BB, BY, and YY; n = 1, 11, and 30, respectively), as shown in Fig. 3*b*. Therefore, the ABO genotype could be determined from the fluorescence ratios of the two probe sets by setting threshold values between each genotype group.

In the aforementioned analyses, we detected a positive signal for the D17Z1 probe (data not shown). To verify the specificity of this probe, we further applied this method to DNA samples derived from the blood of 10 animal species. Although fluorescent signals on the probe sets for ABO genotyping were observed from three primate species, we detected no signals for D17Z1 from all samples (Table 4).

We next applied the DNA chip method to various forensic samples, such as tissue from cadavers, postmortem blood, and blood stains. To determine the genotype groups for the O or B alleles in each individual, we first set the threshold values of the fluorescence ratio based on the results shown in Fig. 3. For the [261 (O)/261 (A, B)] ratio, we set the threshold between OO and XO to 6.5, and the threshold between XO and XX to 2.5 (dashed lines in Fig. 3*a*). Similarly, for the [930 (B)/930 (A, O)] ratio, we set the threshold between BB and BY to 3.0, and the threshold between BY and YY to 1.1 (dashed lines in Fig. 3*b*). On the basis of these thresholds, we used the DNA chip method to determine the ABO

TABLE 3—*Primer sequences.*

Primer	Sequence
ABO1-f	5′-agctcagcttgctgtgtgtt-3′
ABO1-r	5′-agatgctgcatgaatgacc-3′
ABO2-f	5′-gcctgccttgcagatacgtg-3′
ABO2-r	5′-cagagtttacccgttctgct-3′
D17Z1-f	5′-ttttgcaggatctacaagtgga-3′
D17Z1-r	5′-aagaggtctacatgtccccttg-3′

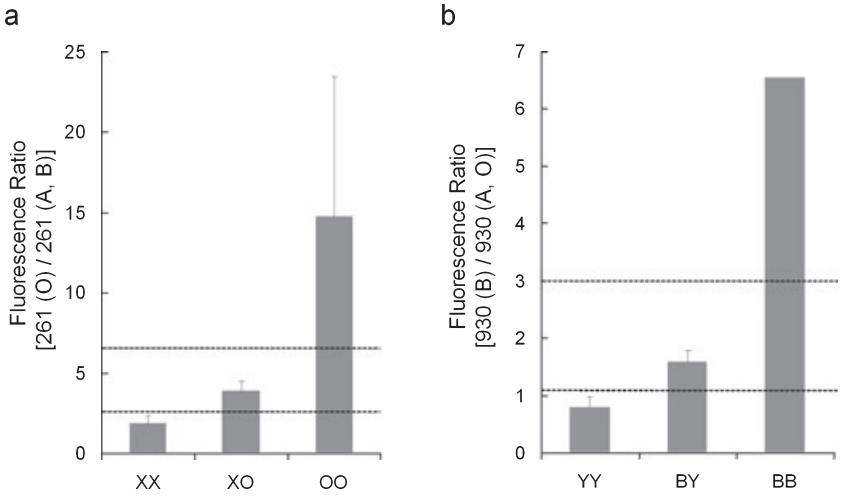

FIG. 3—*Variation in the fluorescence ratio in each probe set. (a) Fluorescence ratio of [261 (O)/261 (A, B)] in OO* (n = *15), heterozygous genotypes for the O allele (XO,* n = *17), and other genotypes (XX,* n = *10). (b) Fluorescence ratio of [930 (B)/930 (A, O)] in BB* (n = *1), heterozygous genotypes for the B allele (BY,* n = *11), and other genotypes (YY,* n = *30). Each bar represents the mean ± SD. The dashed lines in each graph indicate the threshold values for the ABO genotyping used in Table 5.*

genotypes of the forensic samples. All of the results were consistent with the actual ABO genotypes analyzed by the sequencing method (Table 5). Furthermore, we observed a positive signal for the D17Z1 probe in all analyses (data not shown).

Although this study demonstrated that the ABO genotype can be correctly determined by the DNA chip method, several problems, such as the sensitivity and the effect of inhibitory factors, remain to be resolved. In addition, the threshold values between each genotype group should be examined in more detail. Therefore, we will further verify the threshold values, especially between the BB and BY genotypes, using more specimens, as we only had access to one BB genotype sample. Moreover, it is possible that the DNA chip method cannot be applied to degraded samples because of the large size of the amplified fragments. We are currently developing a protocol in which the amplified fragment lengths are less than 100 bp. This method also uses a high number of PCR cycles to facilitate ABO genotyping from low copy number DNA samples. However, the use of high numbers of PCR cycles can generate concomitant problems, such as the strong effect of contamination from other samples. Therefore, these problems need to be further examined in future studies.

This method is expected to be applicable to almost all individuals, because the major O and B alleles have a single nucleotide deletion (261G/Δ) and a single nucleotide substitution (930G/A), respectively; however, there are many low-frequency alleles of the ABO gene, some of which are expected to be falsely genotyped by this method. For example, the B allele B102, which is observed at a frequency of 2.1% in the Japanese population (23), has the same nucleotide as the major A and O alleles at the 930G/A SNP; another B allele–specific single nucleotide substitution is also observed in this allele. Because the 261G/Δ SNP in B102 is the same as observed in the major A and B alleles, this allele would be determined as an ''A allele'' by this method. Such a discrepancy between phenotype and genotype is also problematic when the ABO blood group is determined using other PCR-based methods, including PCR-RFLP. The DNA chip method generally has a greater advantage for the simultaneous analysis of many SNPs; therefore, in this method, the risk a of phenotype–genotype discrepancy can be considerably reduced by adding further probe sets for many rare alleles to the chip. We are currently characterizing further DNA probe sets to detect other B allele–specific nucleotide substitutions, as shown in Table 1.

PCR-based ABO genotyping methods can also detect homologous genes of nonhuman species. In the DNA chip method, we observed positive signals for the ABO probes when we tested

TABLE 4—*DNA chip analyses of various animal samples.*

Species	Fluorescence ratio [261 (O)/261 (A, B)]	Fluorescence ratio [930 (B)/930 (A, O)]	D17Z1
Orangutan	0.22	1.07	N.D.
Chimpanzee	1.41	0.87	N.D.
Green monkey	1.63	0.53	N.D.
Cattle	N.D.	N.D.	N.D.
Japanese serow	N.D.	N.D.	N.D.
Dog	N.D.	N.D.	N.D.
Cat	N.D.	N.D.	N.D.
Squirrel	N.D.	N.D.	N.D.
Norway rat	N.D.	N.D.	N.D.
Chicken	N.D.	N.D.	N.D.

N.D., not detected.

TABLE 5—*ABO genotyping of various forensic samples by the DNA chip method.*

Case no.	Material	Fluorescence ratio [261 (O)/261 (A, B)]	Genotype group XX < 2.5 < XO < 6.5 < OO	Fluorescence ratio [930 (B)/930 (A, O)]	Genotype group YY < 1.1 < BY < 3.0 < BB	Chip result	Known genotype
1	BS(1)	6.18	XO	0.01	YY	AO	AO
2	BS(1)	5.58	XO	1.85	BY	BO	BO
3	BS(1)	15.9	OO	0.71	YY	OO	OO
4	BS(3)	2.17	XX	0.45	YY	AA	AA
5	BS(3)	4.11	XO	1.93	BY	BO	BO
6	BS(3)	2.16	XX	1.77	BY	AB	AB
7	BS(5)	4.33	XO	0.93	YY	AO	AO
8	BS(5)	6.14	XO	2.15	BY	BO	BO
9	BS(5)	13.6	OO	0.85	YY	OO	OO
10	BS(10)	4.46	XO	1.01	YY	AO	AO
11	BS(10)	4.83	XO	1.45	BY	BO	BO
12	BS(10)	9.7	OO	0.42	YY	OO	OO
13	BS(10)	2.26	XX	1.55	BY	AB	AB
14	AH	3.04	XO	0.64	YY	AO	AO
15	AH	3.69	XO	1.48	BY	BO	BO
16	Dao	3.32	XO	0.66	YY	AO	AO
17	Dao	9.53	OO	0.82	YY	OO	OO
18	Dao	1.77	XX	1.46	BY	AB	AB
19	DB	3.76	XO	0.89	YY	AO	AO
20	DB	2.08	XX	1.53	BY	AB	AB
21	DB	4.39	XO	0.82	YY	AO	AO
22	DB	11.17	OO	0.98	YY	OO	OO
23	DB	4.05	XO	1.56	BY	BO	BO
24	DB	0.8	XX	2.1	BY	AB	AB
25	DB	3.78	XO	1.54	BY	BO	BO
26	DB	4.36	XO	0.79	YY	AO	AO
27	DB	19.1	OO	0.98	YY	OO	OO
28	DB	2.96	XO	1.46	BY	BO	BO
29	DB	7.67	OO	0.95	YY	OO	OO
30	DB	2.69	XO	0.67	YY	AO	AO
31	DH	10.21	OO	0.75	YY	OO	OO

BS, blood stain (each parenthetic figure indicates the number of years since it was formed); AH, hair of a living person; Dao, aorta from a cadaver; DB, blood from a cadaver; DH, hair from a cadaver.

DNA samples from primates (orangutan, chimpanzee, and green monkey) (Table 4). On the other hand, although the D17Z1 alpha satellite is primate specific, D17Z1 probe signals were not detected in any primate species other than humans, probably because the primer sequences and/or probe sequence are human specific. Therefore, this method does not detect sequences from nonhuman species and can generate reliable ABO genotyping from biological samples.

As this method requires no special knowledge or skill and takes as little as 4 h from the collection of samples to the generation of results, ABO genotyping with a DNA chip can be quickly and easily performed. Therefore, this DNA chip method meets the recent need for the development of rapid and convenient method of sample typing in criminal investigations.

Conflict of interest: The authors have no relevant conflicts of interest to declare.

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