TECHNICAL NOTE

Zaw Tun,[1] M.B., B.S.; Katsuya Honda,[2] Ph.D.; Masato Nakatome,[3] B.D.S.; Mohammed Nasimul Islam,[4] M.B., B.S.; Hongcheng Bai,[5] M.D.; Yoshiaki Ogura,[6] B.Sc.; Hisanaga Kuroki,[7] M.D.; Motohiko Yamazaki,[8] Ph.D.; Masaru Terada,[9] Ph.D.; and Choei Wakasugi,[10] Ph.D.

Rapid and Clear Detection of ABO Genotypes by Simultaneous PCR-RFLP Method

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ABSTRACT: We reported a new approach of ABO genotyping by a polymerase chain reaction and restriction fragment length polymorphism method. Instead of amplifying the loci containing the positions of nucleotides 258 and 700 of cDNA of the A transferase separately, we successfully amplified these 2 loci together in one reaction mixture using 2 sets of primers. The amplified DNA products were digested at the same time with restriction enzymes *Kpn* I and *Alu* I. The digested DNA products were then separated by electrophoresis on polyacrylamide gel. In addition, we evaluated the influence of various amplification parameters (concentration of template DNA, primers, *Taq* DNA polymerase, $MgCl_2$, and number of cycles). In particular, high Mg^{2+} concentration (3.5 mM) made effective amplification of this locus without producing any unspecific band. By using that optimized condition for PCR, together with a simultaneous approach, our study proved to be time saving, more economic, and convenient in interpreting the results.

KEYWORDS: forensic science, ABO blood-group system, glycosyl transferases, PCR-RFLP, DNA, restriction enzymes

Individual discrimination of unidentified biological samples by ABO blood grouping is the first, easiest, and useful laboratory investigation because ABO blood group is known by everybody before any legal incidents occur. Because the molecular and genetic basis for the ABO blood types has been determined (1,2), many reports on PCR-based ABO genotyping have been published (3–9), but the procedures described are cumbersome, time consuming, and inconvenient in interpreting the results (4,5,6). Here, we report a new approach of ABO genotyping by the PCR-RFLP procedure to reduce these disadvantages.

[1]Postgraduate student, [2]Associate professor, [3]Dental surgeon, [4]Postgraduate student, [5]Postgraduate student, [6]Forensic technician, [7]Research assistant, [8]Research assistant, [9]Forensic toxicologist, and [10]Professor, respectively, Department of Legal Medicine, Osaka University Medical School, Osaka, Japan.

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Materials and Methods

DNA Extraction

Genomic DNA was extracted from serologically determined (10) ABO-known peripheral blood samples according to the standard procedures described (11).

PCR Amplification

Sequences of primers used were as follows (5):

primer 1: 5′-CACCGTGGAAGGATGTCCTC-3′
primer 2: 5′-AATGTCCACAGTCACTCGCC-3′
primer 3: 5′-TGGAGATCCTGACTCCGCTG-3′
primer 4: 5′-GTAGAAATCGCCTCGTCCTT-3′

The standard method of amplification described for the ABO gene locus was as follows (5): Primers (1 + 2) were used for amplification of the 200-bp fragment including the 258th nucleotide and (3 + 4) for 128-bp fragment including the 700th nucleotide. The reaction mixture 100 μL contained 50 ng of template DNA, 200 μM of dNTP, 2.5 units of *Taq* DNA polymerase (Promega, USA), 15 pmole of each primer, and 10 μL of reaction buffer (10 mM Tris-HCl pH 8.3 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100). A total of 35 cycles of amplification were carried out, but the conditions were varied with each set of primers: primer 1 + 2, 94°C denaturation (2 min), 55°C annealing (2 min), 72°C extension (3 min); primer 3 + 4, 94°C denaturation (2 min), 58°C annealing (2 min), and 72°C extension (3 min).

In our study, we selectively varied the amplification parameters as follows (only one parameter was varied in each step):

- Template DNA from 5 ng up to 30 ng,
- *Taq* DNA polymerase from 0.5–3 U in 0.5 U steps,
- Primer concentration from 5–30 pmole,
- Mg^{2+} at a final concentration from 1.5–4 mM, and
- Number of cycles from 15–40.

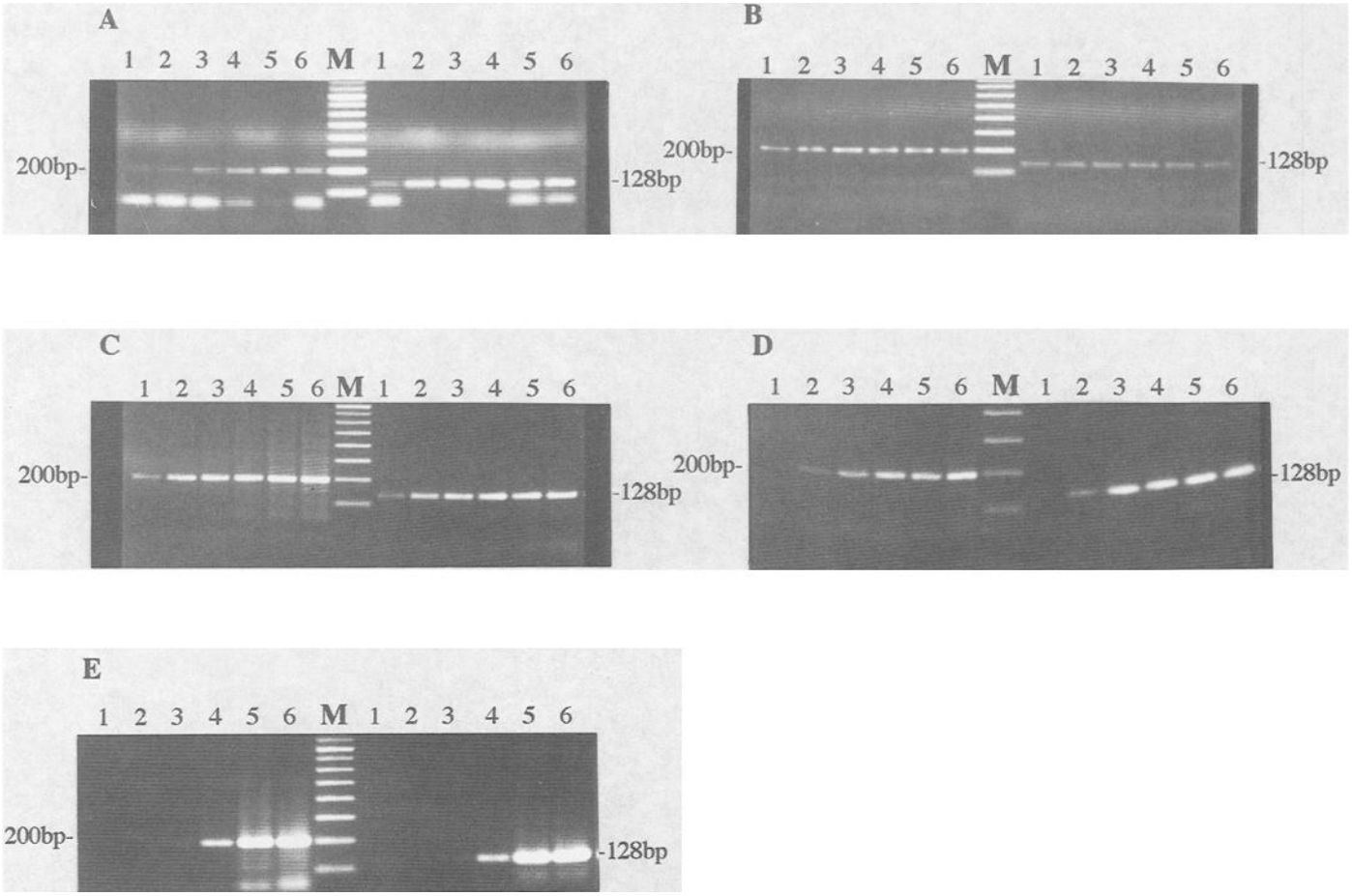

FIG. 1—*Optimization study on amplification of the ABO locus. Ten μL aliquots of amplified products in 50 μL reaction mixture were run in 1% agarose gel electrophoresis. The 200-bp fragment is the product of primers 1 + 2. The 128-bp fragment is the product of primers 3 + 4.* A. *Variation of template; 1 = 5ng, 2 = 10ng, 3 = 15ng, 4 = 20ng, 5 = 25ng, 6 = 30ng.* B. *Variation of* Taq *DNA polymerase; 1–6 = 0.5–3U in increasing order of 0.5U steps.* C. *Variation of primers; 1–6 = 5–30pmol in increasing order of 5pmol steps.* D. *Variation of* Mg^{2+}*; 1 = 1.5mM, 2 = 2mM, 3 = 2.5mM, 4 = 3mM, 5 = 3.5mM, 6 = 4mM.* E. *Variation of cycle numbers; 1 = 15, 2 = 20, 3 = 25, 4 = 30, 5 = 35, 6 = 40.* M. = *100 base-pair ladder.*

Simultaneous Amplification

We performed PCR amplification in a total volume of 50 μL containing the parameters found to be optimal (see below); and each reaction mixture contained all 4 primers. This simultaneous amplification for both O and B allele specific fragments was carried out using a DNA Thermal Cycler (Perkin-Elmer Cetus; PJ 480) according to the following reaction cycles: after initial denaturation at 94°C for 2 min, 30 cycles of 94°C 30 denaturation (1 min), 56°C primer annealing (1 min) and 72°C chain extension (1 min, 30 s), with a final 72°C extension of 5 min.

Enzyme Digestion and Electrophoresis

The amplified products were checked by electrophoresing in 1% agarose gel (FMC Bio Products) for 2 h at 150 V, and direct visualization of the products with ethidium bromide under uv light. Then, 10 μL each of amplified products (which contained 200- and 128-bp fragments) together with 5 units each of restriction enzyme *Kpn* I and *Alu* I were put into the same tube and incubated at 37°C for 1 and a half hour. Digested amplified DNA fragments were electrophoresed on 10% polyacrylamide gel (2 h at 200 V), and the bands visualized after staining with ethidium bromide under uv light. A 100 base-pair ladder (Pharmacia -LKB) was used as a size marker for estimation of fragment sizes.

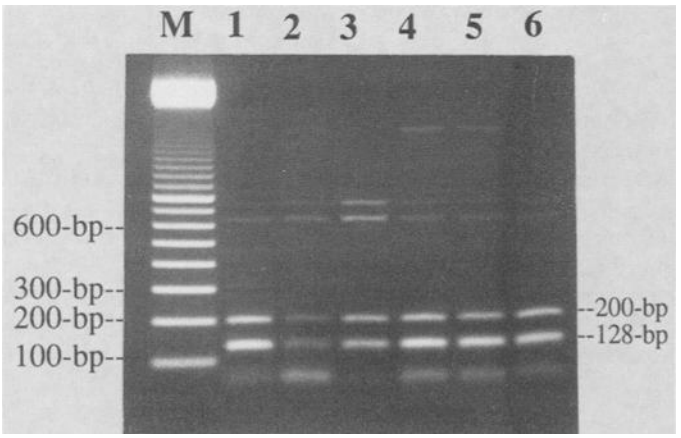

FIG. 2—*Electrophoretic pattern of simultaneous amplification products of ABO locus. Primers 1 + 2 amplified a 199- or 200-bp fragment containing the 258th nucleotide, and primers 3 + 4 amplified a 128-bp fragment containing the 700th nucleotide. Unspecific bands were also seen above the 600-bp. Amplified products were electrophoresed in 1% agarose gel and direct visualization with ethidium bromide under uv light.* M: *100 base-pair ladder; Lanes 1–6: Amplification products for each ABO genotype, 25 ng each of template DNA was used for all products.*

TABLE 1—*Interpretation of ABO genotypes by PCR-RFLP procedure.*

Amplification with primer 1 + 2, Digestion with RE *Kpn* I			Amplification with primer 3 + 4, Digestion with RE *Alu*I		
Genotype	Pattern	Visible bands*	Genotype	Pattern	Visible band*
OO	Com. digestion	171	OO	No digestion	128
AO	1/2 digestion	200, 171	AO	No digestion	128
AA	No digestion	200	AA	No digestion	128
BO	1/2 digestion	200, 171	BO	1/2 digestion	128, 88
BB	No digestion	200	BB	Com. digestion	88
AB	No digestion	200	AB	1/2 digestion	128, 88

*Fragment of 28-bp produced by *Kpn* I digestion and fragment of 40-bp produced by *Alu* I digestion are not visible.
Com. = Complete.

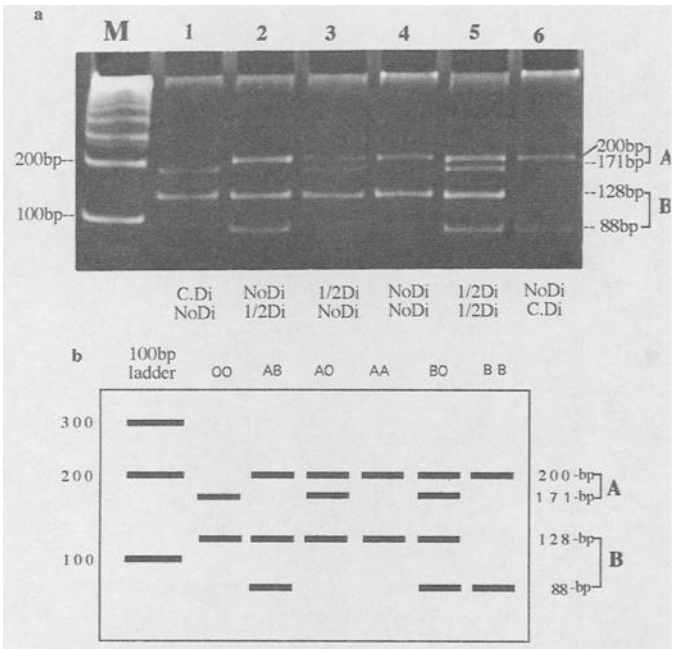

FIG. 3—*Simultaneous RFLP pattern of ABO genotypes from blood samples. Restricted fragments were electrophoresed in 10% polyacrylamide gel and visualized by uv light after ethidium bromide staining.* A: *Digested patterns of 199- or 200-bp fragments.* B: *Digested patterns of 128-bp fragments.* M: *100 base-pair ladder; Lane 1: OO; Lane 2: AB; Lane 3: AO; Lane 4: AA; Lane 5: BO; Lane 6: BB. Each lane consists of* Kpn*I and* Alu *I digests (See Text and TABLE 1).* a: *Photograph of the ethidium bromide stained gel.* b: *Schematic drawing of the banding pattern. C.Di = complete digestion, NoDi = no digestion, 1/2Di = 1/2digestion.*

Results and Discussion

The following parameters were found to be optimal for amplification in 50 μL reaction mixture (Fig. 1): 25 ng of template DNA; 1 unit of *Taq* DNA polymerase (Promega, USA); 10 pmol of each primer; 3.5 mM $MgCl_2$; and 30 reaction cycles. Within a certain range, a change in the above parameters had little or no effect, but beyond this range, fading/disappearance of bands or very strong bands occurred and was associated with either increasing or decreasing concentration (and reaction cycles) of the parameters tested. Generally, failure of amplification was associated with low concentrations (template DNA less than 5 ng, $MgCl_2$ less than 1.5 mM), but we found that a very high concentration of template DNA (over 125 ng) also led to failure.

With the optimized condition for the ABO locus, we could successfully and simultaneously amplify both 200-bp DNA fragments (199-bp in the case of O allele specific fragment) and 128-bp fragments in the same reaction mixture at the same reaction cycles, which was quite different from the method described (5) in which different primer annealing temperature for each set of primers was used. Moreover, we could reduce the duration of these cycles to 50%, and accordingly, it improves the activity of *Taq* DNA polymerase, and amplification was successful even with 1 unit of *Taq* DNA polymerase which is more than 50% lower than the amount needed for conventional amplification. Because we used 4 primers in one reaction mixture for the simultaneous amplification, unspecific bands were occasionally present above the 600-bp (Fig. 2), but they did not cause any effect on further enzyme digestion.

Regarding the enzyme digestion, as Fig. 3 shows, it was possible to digest the 2 different size DNA fragments at the same time because buffer constituents and condition of the RE *Kpn* I and *Alu* I were the same {10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM Dithiothreitol, 37°C, Takara, Japan}. ABO genotypes were determined according to the method previously described (5) as shown in Table 1, but in that, digested pattern of 200-bp DNA fragment had to be compared with its corresponding digested pattern of 128-bp DNA fragment on the other lane to obtain the genotype of ABO blood group. However, by using the present method, one can easily determine the ABO genotype by simply observing the digested patterns of these two fragments in one lane.

The present method has several advantages. First, the major advantage is that the interpretation of the ABO genotype is convenient because the digested pattern of two DNA fragments can be observed in one lane. Second, it is more practical, rapid, and economic because instead of amplifying one sample in two reaction mixtures to obtain the result, we could amplify both fragments in one reaction mixture at same reaction cycles with a reduced amount of *Taq* polymerase. Third, it does not require any special skill or instrument, nor does it require dealing with special electrophoretic separation procedures. Finally, our multiplex-PCR and simultaneous enzyme digestion method may be applicable in experiments in which detection of two or more single nucleotide variations is needed to be examined at the same time.

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Address requests for reprints or additional information to
Zaw Tun
Department of Legal Medicine
Osaka University Medical School
2-2 Yamadaoka, Suita
Osaka 565, Japan