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## Improvement of sensitivity in HLA-DRB1 typing by semi-nested PCR-RFLP.

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## Abstract

A sensitive method of HLA-DRB1 typing was devised using a semi-nested polymerase chain reaction (PCR) followed by a restriction fragment length polymorphism (RFLP) analysis (semi-nested PCR-RFLP method). The first-round amplification (30 cycles) of the semi-nested PCR was performed using DRB generic primer pairs and the second round of PCRs (20 cycles) were performed using DRB1 group-specific primers. The products of the second round PCRs were digested with restriction endonucleases for the typing of HLA-DRB1 alleles. By this method, HLA-DRB1 typing was possible from 10 pg of genomic DNA extracted from lymphocytes and from 0.5 microliter of 1,000 times diluted blood without DNA extraction. HLA-DRB1 alleles could be typed from a 2-mm long bloodstained cotton thread prepared from 10 times diluted blood and from a 2-mm thread of whole blood bloodstains stored at room temperature for 2 years. From the mixture of blood of two individuals with different genotypes, DRB1 alleles of the minor component were detected down to 1/1,000 of the major component. This semi-nested PCR-RFLP method is useful for HLA-DRB1 typing from extremely small amounts of DNA and from mixed samples.

**KEYWORDS:** polymorphism, HLA-DRB1, polymerase chain reaction, dsmi-nested PCR, restriction fragment length polymorphism

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## Improvement of Sensitivity in HLA-DRB1 Typing by Semi-Nested PCR-RFLP

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A sensitive method of HLA-DRB1 typing was devised using a semi-nested polymerase chain reaction (PCR) followed by a restriction fragment length polymorphism (RFLP) analysis (semi-nested PCR-RFLP method). The first-round amplification (30 cycles) of the semi-nested PCR was performed using DRB generic primer pairs and the second round of PCRs (20 cycles) were performed using DRB1 group-specific primers. The products of the second round PCRs were digested with restriction endonucleases for the typing of HLA-DRB1 alleles. By this method, HLA-DRB1 typing was possible from 10 pg of genomic DNA extracted from lymphocytes and from 0.5  $\mu$ l of 1,000 times diluted blood without DNA extraction. HLA-DRB1 alleles could be typed from a 2-mm long bloodstained cotton thread prepared from 10 times diluted blood and from a 2-mm thread of whole blood bloodstains stored at room temperature for 2 years. From the mixture of blood of two individuals with different genotypes, DRB1 alleles of the minor component were detected down to 1/1,000 of the major component. This semi-nested PCR-RFLP method is useful for HLA-DRB1 typing from extremely small amounts of DNA and from mixed samples.

**Key words:** polymorphism, HLA-DRB1, polymerase chain reaction, semi-nested PCR, restriction fragment length polymorphism

The HLA class II region is known to be polymorphic, and detection of the HLA-DQA1 gene has been routinely used for forensic materials. Among HLA class II antigen genes, the DRB1 locus is especially polymorphic, has many alleles as compared with the

DQA1 locus (1), and is therefore thought to be more useful for forensic practice. For HLA-DRB1 typing, methods using the polymerase chain reaction (PCR) have been reported in recent years in the fields of blood transfusion and organ transplantation (1-4). In forensic medicine, there have been some studies using these typing methods (5-8). However, in terms of sensitivity and specificity in forensic practice, there is no satisfactory method which can be used to analyze small amounts or degraded specimens.

Nested PCR has recently attracted attention due to its improvement in both sensitivity and specificity. In forensic medicine, this method has been used for the detection of the Amelogenin (9), HLA-DQA1 (10) or COL2A1 gene (11). Uchihashi *et al.* reported that the semi-nested PCR method has made it possible to amplify a single copy of a gene from a single genome or a single sperm (10).

In this study, we amplified the HLA-DRB1 gene using the semi-nested PCR method. The amplification products were then subjected to HLA-DRB1 typing by a modified restriction fragment length polymorphism (RFLP) analysis, a simple, practical and inexpensive technique, reported by Ota *et al.* (12). We examined the sensitivity and specificity of this semi-nested PCR-RFLP method and evaluated its applicability to forensic specimens.

### Materials

**Materials.** DNA was extracted from peripheral blood lymphocytes (about  $10^6$  cells), 10  $\mu$ l and 1  $\mu$ l of whole blood and 5  $\mu$ l each of its 1/10, 1/100 and 1/1,000 dilutions with TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Whole blood samples from 2 subjects with known DRB1 types were mixed at ratios from 1,000:

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1 to 1:1,000, and DNA extracted from 9  $\mu$ l of the mixture was used. Whole blood samples from 5 subjects with known DRB1 types were diluted 1/10–1/100,000 with TE-buffer. Without DNA extraction, 0.5  $\mu$ l of these dilutions was directly used as a template for PCR. Whole blood samples from 5 subjects with known DRB1 types and their 1/10, 1/100 and 1/1,000 dilutions with TE-buffer were applied to cotton cloths. Bloodstained threads were cut at lengths of 2-mm. Each 2-mm thread was directly used as a template for PCR. The bloodstains prepared from whole blood samples were stored at room temperature. After 5 days, 1 month, 3 months and 2 years, each 2-mm thread was directly subjected to PCR. Whole blood samples (500  $\mu$ l) from 5 subjects with known DRB1 types were placed in tubes, left for 2h, tightly capped, and stored at room temperature in a moist chamber. After 1 day, 3 days, 8 days, 14 days and 1 month, 30  $\mu$ l of each sample was diluted 1/10 with TE-buffer, and 0.5  $\mu$ l of the dilution was directly used as a template for PCR.

**DNA extraction.** To each of the specimens, 500  $\mu$ l of lysis buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 0.1 % Triton X-100, 1 % SDS, 250  $\mu$ g/ml Proteinase K) was added and the mixture was heated at 60°C for 1h. Then, after heating at 95°C for 10 min to inactivate the protease, DNA was recovered by phenol-chloroform extraction and isopropanol precipitation. DNA recovered from lymphocytes, whole blood, diluted blood and mixed blood was dissolved in 100, 20, 20 and 30  $\mu$ l of TE-buffer, respectively, and 0.5  $\mu$ l of each was used as a template for PCR. DNA extracted from lymphocytes was quantified by absorbance at 260nm with a spectrophotometer (Beckman Instruments Inc., Irvine, CA, USA).

**Pretreatment of samples with GeneReleaser™.** GeneReleaser™ (BioVentures, Murfreesboro, TN, USA), which allows the release of template DNA by heating alone and simultaneous removal of PCR inhibitors, was used for pretreatment of samples. Whole blood, diluted blood and whole blood stored at room temperature (1  $\mu$ l each) and bloodstained threads stored at room temperature (about 2mm in length) were mixed with 20  $\mu$ l of well shaken GeneReleaser™. According to the manufacturer's instructions, the mixture was heated and the whole mixture was directly used as a template for PCR.

## Methods

**Principles of HLA-DRB1 typing by semi-nested PCR-RFLP.** The HLA-DRB1 typing method used in this study consisted of two-stage PCR (semi-nested PCR) and RFLP analysis. In the first-stage PCR (first PCR), the 274bp fragment of the DRB gene was amplified by 30 cycles of PCR using DRBAMP-A (5'DRB) and DRBAMP-B (3'DRB), which were the DRB generic primer pair used at the 11th International Histocompatibility Workshop (1). In the second-stage PCR (second PCR), DR1, DR2, DR3568 (DR3), DR4 and DR9, 5 allele groups of the DRB1 gene, were specifically amplified by 20 cycles of PCR using an aliquot of the first PCR amplification product as a template and using a set of 5 pairs of primers from DR1 to DR9 (Tables 1 and 2). Next, HLA-DRB1 typing was performed by RFLP analysis of the second PCR products.

**Amplification and typing of DRB1 gene.** The reaction mixture for the first and second PCR consisted of 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1 % Triton X-100, 1.5 or 2.0mM MgCl<sub>2</sub>, 0.2mM each of dNTPs, each primer at the final concentration of 0.5  $\mu$ M and 0.1U/ $\mu$ l of Taq DNA polymerase (Promega, Madison, WI, USA). When samples other than extracted DNA were used in the first PCR, bovine serum albumin was added to the reaction solution at 200  $\mu$ g/ml to suppress the influence of PCR inhibitors (13–15). The template was added to the reaction mixture (24.5  $\mu$ l/tube), and a drop of mineral oil was added. PCR was performed using a Thermal Cycler PC-800 (ASTECH, Fukuoka, Japan) under the conditions shown in Table 2. In the second PCR, 0.5  $\mu$ l of the first PCR product was used as a template.

**Table 1** Oligonucleotide primers used in the present method

| Primer   | Nucleotide sequence (5' → 3') |
|----------|-------------------------------|
| 5'DRB    | CCCCACAGCACGTTTCTTG           |
| 5'DR1    | TTCTTGTGGGAGCTTAAGTT          |
| 5'DR2    | TTCTGTGGCAGCCTAAGAGG          |
| 5'DR3568 | ACGTTTCTTGGAGTACTCTACG        |
| 5'DR4    | GTTTCTTGGAGCAGGTTAAAC         |
| 5'DR9    | GGACGGAGCGGGTGCGGTATC         |
| 3'DR9    | CCGTAGTTGTGTCTGCACACGG        |
| 3'DRB    | CCGCTGCACTGTGAAGCTCT          |

**Table 2** Semi-nested polymerase chain reaction (PCR) amplification of HLA-DRB1 gene

| PCR                      | Primer set |       | Cycles | Product<br>(Base pair) | References |
|--------------------------|------------|-------|--------|------------------------|------------|
|                          | 5'         | 3'    |        |                        |            |
| 1st PCR (DRB generic)    | 5'DRB      | 3'DRB | 30     | 274                    | (12)       |
| 2nd PCR (Group specific) |            |       |        |                        |            |
| DR1                      | 5'DR1      | 3'DRB | 20     | 261                    | (1)        |
| DR2                      | 5'DR2      | 3'DRB | 20     | 261                    | (12)       |
| DR3568                   | 5'DR3568   | 3'DRB | 20     | 265                    | (12)       |
| DR4                      | 5'DR4      | 3'DRB | 20     | 263                    | (12)       |
| DR9                      | 5'DR9      | 3'DR9 | 20     | 193                    | (23)       |

The first and second PCR products (7  $\mu$ l) were subjected to electrophoresis on a 5% polyacrylamide gel, stained with ethidium bromide, and observed on an ultraviolet transilluminator TDM-30 (UVP, San Gabriel, CA, USA).

Allele typing of the second PCR products was followed by a modified RFLP method reported by Ota *et al.* (12). A DRB1\*0901 allele of the DR9 group was confirmed by Mnl I digestion.

**Paternity test after the death of the putative father.** In a paternity case after the death of the putative father, examination by the present HLA-DRB1 typing method was performed. DNA extracted from peripheral blood lymphocytes of the child, the plaintiff, and the mother was used. The putative father's DNA, extracted from a quarter section (about 0.5 cm<sup>2</sup>) of dried blood preserved within the Rh<sub>0</sub> (D) examination frame on a sheet of 'blood typing paper' attached to a clinical record card (date: December 27, 1991) at the hospital he was admitted to before death, was used.

## Results

**Sensitivity.** The DRB genes were amplified by the first PCR using template DNA (1,000 pg-0.1 pg) extracted from the lymphocytes of 5 subjects in whom the DR type had been determined previously by serological typing. By the first-round PCR alone, the amplification band of the DRB gene could be detected using 1 ng template DNA. Subsequently, the second PCR was performed using an aliquot of the amplification product in the first PCR as a template. In each sample, the DRB1 gene could be detected using 10 pg or more template

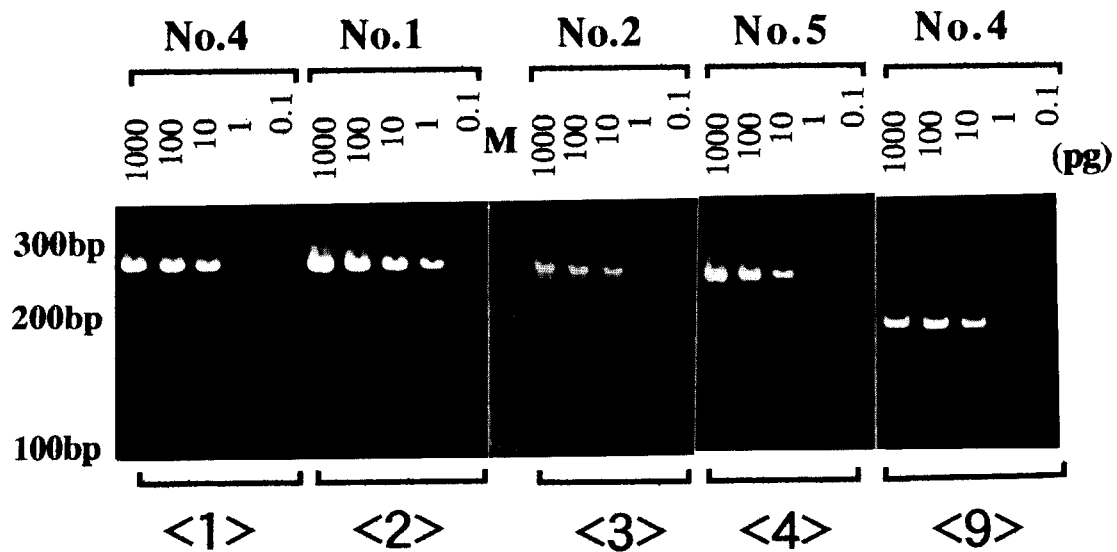
DNA for the first PCR (Fig. 1). The DRB1 gene was amplified by this method using DNA extracted from whole blood or diluted blood with known DRB1 types. Each of the DRB1 allele groups was detected using a 1/40 portion of DNA extracted from 5  $\mu$ l of 1/100 or less blood dilutions (Table 3).

### **DRB1 typing from mixed blood samples.**

Whole blood samples obtained from 2 subjects with different DRB1 types (No. 2: DRB1\*0405/0803, No. 3: DRB1\*0901/1202) were mixed in a ratio of 1,000:1-1:1,000. DNA was extracted from the mixtures, and semi-nested PCR was performed (Fig. 2). For the DR3 group shared by the 2 subjects, an amplification band was detected in all mixture ratios. For the DR4 group derived from sample No. 2, an amplification band was detected in a mixture ratio of 1/1,000 or more. For the DR9 group derived from sample No. 3, an amplification band was detected in a mixture ratio of 1/100 or more. When the amplification products of the DR3 group were analyzed with the restriction enzymes Fok I and Sac II, DRB1\*0803 derived from sample No. 2 was detected in a mixture ratio of 1:1 or more, and DRB1\*1202 from sample No. 3 was detected in a mixture ratio of 1:10 or more.

### **DRB1 typing from diluted blood and bloodstains of diluted blood.**

Whole blood from 5 subjects with known DRB1 types and their 1/10-1/100,000 dilutions were subjected to semi-nested PCR without DNA extraction. The amplification band of the DRB1 gene was detected in the 1/100-1/1,000 blood dilutions and in 4 of the 1/10 dilution samples. It could not be detected in undiluted samples and one of 1/10 diluted sample. However, when the samples were pre-



**Fig. 1** Detection of DRB1 alleles from lymphocyte DNA. The DRB1 gene was amplified by semi-nested polymerase chain reaction (PCR) using template DNA (1,000 pg–0.1 pg) extracted from the lymphocytes of 4 subjects.

No. 1: DRB1\*0101/1502 (Group <1>/<2>); No. 2: DRB1\*0405/0803 (Group <4>/<3>); No. 4: DRB1\*0101/0901 (Group <1>/<9>); No. 5: DRB1\*1502/0405 (Group <2>/<4>).

<1>: amplification products of a DR1 allele group; <2>: amplification products of a DR2 allele group; <3>: amplification products of a DR3 allele group; <4>: amplification products of a DR4 allele group; <9>: amplification products of a DR9 allele group.

M: 100 base pair (bp) ladder

**Table 3** Detection of DRB1 alleles using DNA extracted from whole blood and diluted blood

| PCR     | 1/40 portion of DNA extracted from |   |                            |       |        |
|---------|------------------------------------|---|----------------------------|-------|--------|
|         | Whole blood ( $\mu$ l)             |   | 5 $\mu$ l of blood diluted |       |        |
|         | 10                                 | 1 | 1/10                       | 1/100 | 1/1000 |
| 1st PCR | +                                  | – | –                          | –     | –      |
| 2nd PCR |                                    |   |                            |       |        |
| DR1     | +                                  | + | +                          | +     | –      |
| DR2     | +                                  | + | +                          | +     | +      |
| DR3568  | +                                  | + | +                          | +     | –      |
| DR4     | +                                  | + | +                          | +     | –      |
| DR9     | +                                  | + | +                          | +     | –      |

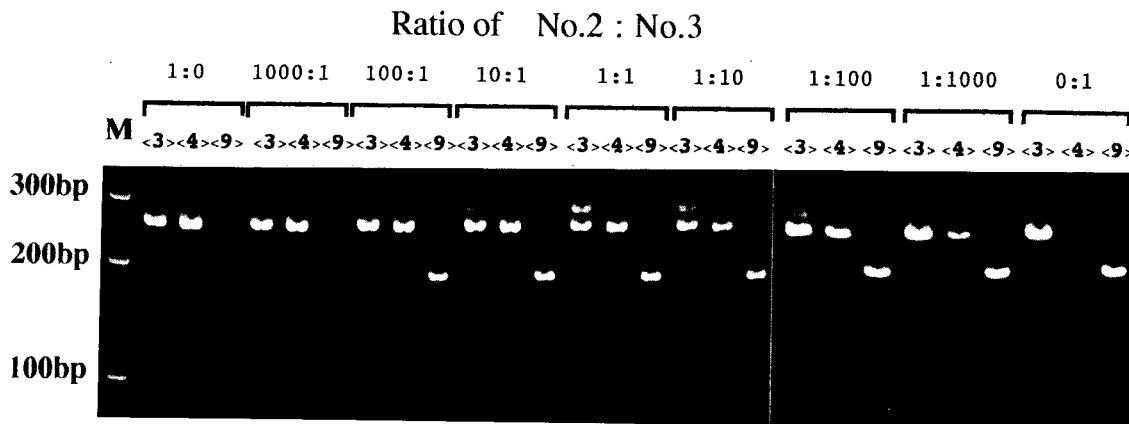
+: Detectable; –: Undetectable. PCR: See legend to Fig. 1.

treated with GeneReleaser™, the DRB1 gene could be detected from these samples. From 1/10,000 or more diluted samples, the DRB1 gene was not detected. When the first round PCR alone was performed, the amplification band could be detected only in 3 of the 1/10 dilution samples and in 4 of the 1/100 dilution samples without DNA extraction. No bands were detected from 2 of the

1/10 diluted samples and undiluted blood.

Bloodstains prepared from whole blood and diluted blood were subjected to semi-nested PCR without DNA extraction. The allele could be detected in a single 2-mm thread with bloodstains of 1/10 diluted blood or whole blood.

**DRB1 typing from decomposed blood and**



**Fig. 2** Detection of DRB1 alleles from the mixture of two individuals' blood with different genotypes. No. 2: DRB1\*0405/0803 (Group <4>/<3>); No. 3: DRB1\*0901/1202 (Group <9>/<3>). <3>: amplification products of a DR3 allele group; <4>: amplification products of a DR4 allele group; <9>: amplification products of a DR9 allele group. M: 100 base pair (bp) ladder

#### **aged bloodstains stored at room temperature.**

Whole blood samples from 5 individuals with known DRB1 types were stored at room temperature and directly subjected to semi-nested PCR. Allele groups corresponding to each type could be detected in samples after 3 or 8 days of storage. In samples after 1 day, 3 out of 5 allele groups, and in samples after 14 days to 1 month of storage, 4 out of 5 allele groups could not be detected. When the same samples after 1 day were treated with GeneReleaser™ and subjected to semi-nested PCR, all allele groups were detected. However, when the same samples after 14 days to 1 month of storage were prepared with GeneReleaser™, bands were not detected in the allele groups.

Bloodstains prepared from whole blood were stored at room temperature, and the threads (2-mm in length) were directly subjected to semi-nested PCR. In samples from 5 subjects with known DRB1 types after 1 month, 3 months and 2 years of storage, all the corresponding allele groups were detected. In samples after 5 days, no amplification bands were detected in 2 out of 5 samples.

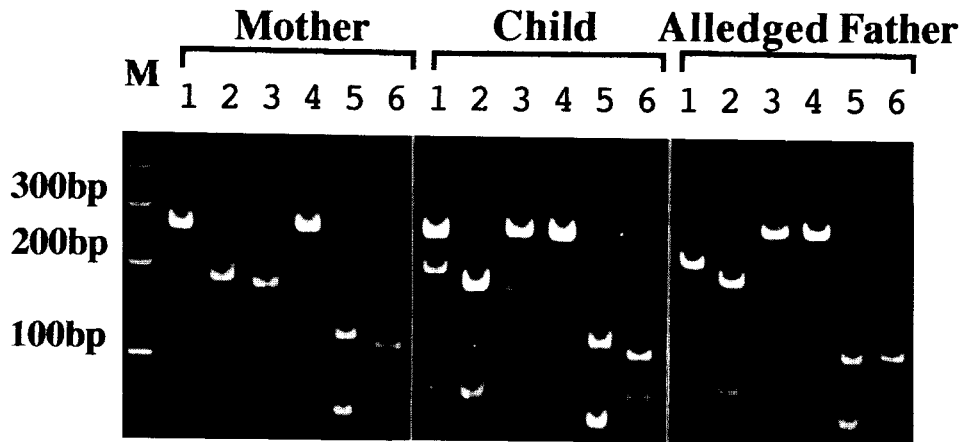
**DRB1 typing in paternity cases.** In a paternity case after the death of the putative father, examination of DNA extracted from lymphocytes revealed alleles in DR4 and DR9 in the mother, and DR4 in the child. Furthermore, examination of DNA extracted from dried blood on the 'blood typing paper' revealed alleles in DR4 and DR9 groups in the putative father. In addition,

allele typing by the modified-RFLP showed DRB1\*0406/0901 in the mother, DRB1\*0401/0406 in the child, and DRB1\*0401/0901 in the putative father (Fig. 3). These findings were consistent with paternity. In this case, when the results of DRB1 typing were applied to Essen-Möler's formula, the probability of paternity was 93.06 %.

In the other 9 ordinary paternity cases in which paternity relationships were not excluded in 6 cases and excluded in 3 cases by the examination of the conventional 22 blood type systems, no genetic contradiction was found with results of HLA-DRB1 typing (Table 4). In 1 of these 3 excluded cases (#081119), however, a paternity relationship was not excluded by the HLA-DRB1 typing.

#### **Discussion**

In this study, we devised a sensitive method for HLA-DRB1 typing by using the semi-nested PCR followed by a modified RFLP analysis. The DRB1 gene could be detected from 10 pg of template DNA by the present method. Concerning the high sensitivity of nested-PCR, Uchihi *et al.*, who detected the HLA-DQA1 gene by semi-nested PCR, reported detection of the gene using 3 pg or more of template DNA (10). Honda *et al.*, who detected the COL2A1 3' variable region (11) and the MCT118 (D1S80) region (16) by nested-PCR, reported



**Fig. 3** DRB1 genotyping in a paternity case after the death of the putative father. PCR products of a DR4 group were digested with the following restriction endonucleases: 1: Sac II; 2: Ava II; 3: Hinf I; 4: Hae II; 5: Hph I; 6: Mnl I  
M: 100 base pair (bp) ladder

**Table 4** HLA-DRB1 typing in 9 ordinary paternity cases

| Cases                                 | DRB1 types of |             |                |
|---------------------------------------|---------------|-------------|----------------|
|                                       | Mother        | Child       | Alleged father |
| <b>Non-excluded cases<sup>a</sup></b> |               |             |                |
| #090120                               | *0101/*0901   | *0101/*0901 | *0101/*1103    |
| #081111                               | *0405/*0901   | *0405/*0405 | *0802/*0405    |
| #080708                               | *1101/*0406   | *1101/*1302 | *1302/*0405    |
| #060321                               | *0901/*0901   | *1302/*0901 | *1302/*0901    |
| #060209                               | *0405/*0901   | *1302/*0901 | *1302/*0405    |
| #060120                               | *0101/*0901   | *0101/*0901 | *0403/*0901    |
| <b>Excluded cases<sup>b</sup></b>     |               |             |                |
| #090424                               | *1302/*0405   | *1501/*0405 | *1202/*1302    |
| #081119 <sup>c</sup>                  | *1101/*1201   | *1101/*0405 | *1202/*0405    |
| #060322                               | *0901/*0901   | *1302/*0901 | *1501/*1502    |

<sup>a</sup>: Paternity was not excluded by the examination of the conventional 22 blood type systems.

<sup>b</sup>: Paternity was excluded by the examination of the conventional 22 blood type systems.

<sup>c</sup>: Paternity was excluded by the examination of the conventional 22 blood type systems, but it was not excluded by the DRB1 typing.

detection of these genes using 5 pg or more and 10 pg or more of template DNA, respectively. The present HLA-DRB1 typing method by semi-nested PCR has high sensitivity comparable to the sensitivity reported by these authors.

In the experiment of mixed blood samples from two subjects, when the subjects had different alleles in the same allele group, the minor component allele was detected in a mixture ratio of 1:10 or more. When the alleles in the two subjects belonged to different allele groups, the

minor component allele could be detected in a mixture ratio of 1:100–1:1,000. Gyllenstein *et al.* performed HLA-DQA1 typing of mixed samples by allele-specific PCR analysis and reported detection of the minor component allele in a mixture ratio of 1:10,000 or more (17).

In amplification of alleles from individuals in mixed samples by routine PCR without allele group-specific primers, competition between templates and primers occurs, and the minor component ratio in amplified products depends on its initial proportion in the mixture



sample (18, 19). Therefore, in typing of STR (short tandem repeats, microsatellite) or VNTR (variable number of tandem repeats) polymorphism, detection of the minor component allele that is present in a mixture ratio of 1/100 or less is impossible by PCR using primers common to all alleles (20-22). On the other hand, by using PCR with allele-group-specific primers for HLA-DRB1 typing in this study, the detection state was similar to that by routine PCR when individual alleles in mixed samples belonged to the same allele group (the DR3 group in this study). However, when the minor component allele and the major component allele belonged to different allele groups, the minor component allele, even in a mixture ratio of 1/1,000, could be detected due to the absence of competition between templates.

The DRB1 gene could be detected in the 1/100 or more diluted blood samples, but it could not be detected in whole blood and some of the 1/10 diluted blood samples by semi-nested PCR. This was most likely caused by PCR inhibitors in blood (13-15). When these samples were pretreated with GeneReleaser™, the DRB1 gene became detectable. Although the 1/100 or more diluted samples may be negligibly affected by these inhibitors, amplification products required for DRB1 typing were difficult to obtain from these samples by the first-round PCR alone because of the insufficient amount of template DNA. However, after semi-nested PCR, which requires only a small amount of template DNA, a sufficient amount of the DRB1 gene could be amplified even from the 1/1,000 dilution samples, which were negligibly affected by the inhibitors.

The cause of the failure in detection from blood samples after 1 day and bloodstains after 5 days may be due to the fact that the PCR inhibitors in blood affected the amplification more seriously during the early stages of storage. When these samples were pretreated with GeneReleaser™ and directly subjected to semi-nested PCR, all the corresponding allele groups were detected.

The success in the paternity testing using an aged blood clot on the 'blood typing paper' as the test material suggests the usefulness of the present method for HLA-DRB1 typing in forensic applications.

In three ordinary paternity cases in which paternity relationships were excluded by the conventional blood markers, HLA-DRB1 typing could not exclude paternity in one case. This may be caused by the fact that the chance exclusion of paternity for HLA-DRB1 typing by the present method is not equivalent to that calculated for

the conventional blood markers. In the situation, it is more accurate to perform the present HLA-DRB1 typing and typing of microsatellite markers at the same time.

In conclusion, HLA-DRB1 typing by the semi-nested PCR-RFLP method in this study increased the sensitivity 10-100 times over conventional PCR and allowed typing of the minor component contained in mixed samples at a ratio of 1/1,000. Therefore, this method is useful for typing an extremely small sample or mixed blood. This method may be applicable to the determination of the assailant from vaginal swabs in sexual crimes and in the demonstration of heterogeneity in incompatible blood transfusions.

Furthermore, the semi-nested PCR-RFLP method in this study has the following advantages: Since samples are directly subjected to amplification reactions without DNA extraction, contamination during extraction does not occur. Using the small aliquot of PCR products obtained by the first round, sufficient amounts of amplification products required for RFLP analysis can be obtained by the second round PCR. Since the amount of template for the second PCR can be adjusted according to the amplification state of the DRB genes by the first PCR, typing is possible from a wide range of sample DNA quantities available. In forensic medicine, in which examination results are of great importance, the HLA-DRB1 typing method presented here, while slightly complicated, is useful because of its high reliability.

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