TECHNICAL NOTE

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Validation of HLA-DR Locus Typing in Forensic Specimens by Combining PCR-SSP with PCR-RFLP

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ABSTRACT: The applicability of HLA-DR DNA typing combined with PCR-SSP(sequence specific primers) and PCR-RFLP(restriction fragment length polymorphism) to forensic practice was investigated. PCR-SSP was as effective as serological HLA-DR typing in determining DR types. For more precise definition of DRB1 alleles encoding DR2, DR4, and DR8 antigens, which are fairly common in Japan, we used the PCR-RFLP method. For increasing the sensitivity of this system, we used the nested or semi-nested PCR technique. The minimum amount of template DNA needed for typing was 10 ng of genomic DNA in the case of ordinary PCR, whereas 10 pg of DNA was enough in nested and semi-nested PCR. HLA-DR and-DRB1 alleles were able to be determined from the small amounts of DNA available in forensic materials using the PCR-SSP and subsequent PCR-RFLP methods.

KEYWORDS: forensic science, HLA-DNA typing, DRB1 allele, PCR-SSP, PCR-RFLP, forensic samples

An analysis of the HLA complex by molecular methods has revealed a high number of polymorphisms (1). These genes are useful human genetic markers in forensic investigations. Class II HLA polymorphisms have been defined at the DNA level, due to the availability of allelic nucleotide sequences in this region as well as the successful application of molecular typing techniques. Various PCR based HLA class II DNA typing methods have been developed and applied (2–6). To date, one commercial kit for HLA-DQA1 typing has been validated for forensic investigations (7), and is used routinely in many laboratories. Among HLA class II genes, the DRB1 gene is one of the most useful in forensic genetics because it has many alleles, 106 kinds of DRB1 alleles (8) have been identified using PCR based methods up to now. If sufficient high-quality DNA are recovered from forensic samples, definition for all DRB1 alleles is possible by PCR-SSP, PCR-SSO (sequence specific oligonucleotide), PCR-RFLP, PCR-SBT (sequence based typing), and other PCR-based methods. However, except in extraordinarily well preserved samples, we generally have only an extremely small amounts of DNA from a single hair, a piece of nail, formalin-fixed and paraffin-embedded tissues and so on. Therefore, to accommodate this condition, we attempted to apply PCR-SSP and subsequent PCR-RFLP methods to forensic samples. The PCR-SSP technique using allele specific primers is capable of typing a low degree of polymorphisms corresponding to serologically identified DR antigens (low-resolution) and a high degree of polymorphisms on DRB1 alleles (high-resolution). This PCR-SSP is also simple and easy to perform. In this study, we have investigated the applicability and the level of sensitivity of HLA-DR "low-resolution" typing by PCR-SSP and subsequent PCR-RFLP using nested or semi-nested PCR for forensic practice.

Materials and Methods

Samples

Blood samples were taken from 35 healthy unrelated Japanese whose serological DR type were known. Eleven kinds of homozygous B cell lines, provided by the 10th International Histocompatibility Workshop were used in this study. One tooth obtained at a forensic autopsy and stored at −20°C for seven years, 5 cm of the root portion of the hair shaft stored in a dedicator for up to five years, and 10 μL of fresh bloodstain from a cotton sheet were used in this study.

DNA Extraction

DNA from whole blood was extracted using the following method (9). After the samples were incubated overnight in a lysis buffer (100 mM Trs-HCl, 200 mM NaCl, 40 mM $EDTANa_2$ and 2% SDS, pH 8.0), 0.2 mg/mL proteinase K at 50°C. DNA was precipitated with absolute ethanol after extraction with 400 mM Tris saturated phenol.

DNA from the tooth was obtained using the same phenol extraction and alcohol precipitation method after powdering the sample in a freezer mill (Speck Inc., NJ, USA).

The 5-cm hair was cut into five pieces approximately 1 cm in length. The pieces were washed in 100% ethanol and then briefly rinsed in sterile distilled water in a 1.5 mL micro centrifuge tube. After removing water they were soaked in 200 μL of lysis buffer with 0.2 mg/mL proteinase K and incubated overnight at 50°C. Phenol/chloroform/isoamyl alcohol (25:24:1; 200 μL) was added

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to tube. Tube was then rotated at 400 rpm for 30 min, and spun in a microcentrifuge for 20 min at 15,000 rpm. The aqueous phase was removed and transferred to a Centricon 100 devices (Amicon Co., MA, USA) (10). The sample volume in Centricon was brought to approximately 1 mL by adding TE buffer. The DNA extract was dialyzed and concentrated by centrifuging the Centricon for 20 min at 2500 rpm. This dialysis and concentration procedure was repeated with sterile distilled water.

DNA from bloodstains was purified with a combination of Chelex 100 cheleting resin (Bio-Rad) and Centricon 100 dialysis (11).

The concentration of DNA in these extract was quantified by optical density at 260 nm (OD_{260}) minus OD_{320}, and their purity was checked by the ratio OD_{260}/OD_{280} (12).

PCR Primers—To define HLA-DR1 to DR10 by PCR-SSP, we designed 10 forward primers and 9 reverse primers (Table 1), which were based on those in previous publications (3,4), with some modifications (3'R3 and 3'R14-2).

Amplification Conditions—The PCR reactions were carried out in 25 μL volumes, which contained genomic DNA template, 10 pmol of each sequence-specific primer, 200 μM of each dNTP, 2 units of Taq DNA polymerase, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH8.3, 50 mM KCl, and 0.1% Triton-X. Samples were amplified after initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C denaturation for 60 s, 60°C annealing for 60 s, 72°C extension for 90 s, and a final 72°C extension for 5 min, using the thermal sequencer TSR-300 (Iwaki Glass Co., Japan).

Nested PCR

In order to type from extremely small amounts of DNA, we applied the nested PCR method to increase amplification sensitivity. Generic primers were generated from outside sequences to the nested primers used for PCR-SSP (Table 1). The first round PCR amplification was performed using generic primers (DRBF and DRBR). The PCR reaction mixture (50 μL) contained PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl pH8.3, 50 mM KCl, and 0.1% Triton-X), genomic DNA template, 10 pmol of each generic primer, 200 μM of each dNTP, 2 units of Taq DNA polymerase. Samples were amplified after initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C denaturation for 60 s, 60°C annealing for 60 s, 72°C extension for 90 s, and a final 72°C extension for 5 min, using the thermal sequencer TSR-300. After performing the first round of PCR, the amplification mixture was purified with a Centri-Sep column (Princeton Separations, Inc. NJ, USA) to remove excess primers and dNTPs according to the manufacture's instructions. The eluate was dried in a vacuum centrifuge. The DNA sample was dissolved in an appropriate volume (20 to 40 μL) of sterile distilled water. One μL of purified DNA was usually subjected to a second round of PCR. The second PCR was performed under the following conditions: 1 μL of the first amplification product was used as the template in 100 μL of reaction mixture. Constituents and concentrations of the reaction buffer and reaction thermocycles were the same as in the first round of PCR, except that the number of cycles was reduced to 20.

Visualization of Amplifications

PCR products using SSP were subjected to electrophoresis in 2% agarose gels in a horizontal minigel apparatus (Mupid, Cosmo Bio Co. Ltd.). Absence or presence of PCR products was visualized by staining gels with ethidium bromide. To determine sub-alleles for DR2, DR4 or DR8 using PCR-RFLP method, samples of the restriction enzyme-cleaved amplified DNAs were subjected to electrophoresis in 12% polyacrylamide gels in a horizontal minigel apparatus. Cleavage or no cleavage of amplified fragments was detected by staining with ethidium bromide. The cleavage patterns of PCR products with restriction enzymes were mentioned elsewhere (3).

Results and Discussions

To evaluate the efficiency of SSP (DR1 to DR9 primers), PCR amplification was carried out using DNA samples extracted from homozygous B cell lines of the 10th International Histocompatibility Workshop. Figure 1A shows the results of electrophoresis of PCR amplification products. The appropriate length (Table 1) of products could be amplified with each primer pair (DR1: 5'R1/DRBR; 206bp, DR2: 5'R2/DRBR; 261bp, DR3: DR3/3'R3; 150bp, DR4: 5'R4/DRBR, DR11: 5'R3568/3'R11; 178bp, DR12: 5'R12/

TABLE 1—*Primer pairs for identification of DR1-DR10 specificities by PCR-SSP technique.*

G.P.	5' primer DRBF:5'-CCGGATCCTTCGTGTCCCCACAGCACG	3' primer DRBR:5'-CCGCTGCACTGTGAAGCTCT	Amplified Fragments 289 (bp)
SSP			
DR1	5'R1: 5'-GGTTGCTGGAAAGATGCATCT	DRBR: 5'-CCGCTGCACTGTGAAGCTCT	206(bp)
DR2	5'R2: 5'-TTCCTGTGGCAGCCTAAGAGG	DRBR: 5'-CCGCTGCACTGTGAAGCTCT	261(bp)
DR3	5'R3: 5'-TACTTCCATAACCAGGAGGAGA	3'R3: 5'-AGTAGTTGTCCACCCGGC	150(bp)
DR4	5'R4: 5'-GTTTCTTGGAGCAGGTTAAAC	DRBR: 5'CCGCTGCACTGTGAAGCTCT	263(bp)
DR11	5'R3568:5'-ACGTTTCTTGGAGTACTCTACG	3'R11: 5'-CTGGCTGTTCCAGTACTCCT	178(bp)
DR12	5'R12: 5'-AGTACTCTACGGGTGAGTGTT	3'R12: 5'-CACTGTGAAGCTCTCCACAG	248(bp)
DR13.1	5'R3568:5'-ACGTTTCTTGGAGTACTCTACG	3'R13-1:5'-CCCGCTCGTCTTCCAGGAT	203(bp)
DR13.2	5'R3568:5'-ACGTTTCTTGGAGTACTCTACG	3'R13-2:5'-TGTTCCAGTACTCGGCGCT	173(bp)
DR14.1	5'R3568:5'-ACGTTTCTTGGAGTACTCTACG	3'R14-1:5'-TCTGCAATAGGTGTCCACCT	226(bp)
DR14.2	5'R14.2:5'-TACTTCCATAACCAGGAGGAGA	3'R14-2:5'-TCCACCGCGGCCCGCCT	140(bp)
DR7	5'R7: 5'-AGTTCCTGGAAAGACTCTTCT	DRBR: 5'-CCGCTGCACTGTGAAGCTCT	206(bp)
DR8	5'R3568:5'-ACGTTTCTTGGAGTACTCTACG	3'R8: 5'-CTGCAGTAGGTGTCCACCAG	225(bp)
DR9	5'R9: 5'-GAAGCAGGATAAGTTTGAGTG	DRBR: 5'-CCGCTGCACTGTGAAGCTCT	256(bp)
DR10	5'R10: 5'-GGTTGCTGGAAAGACGCGTCC	DRBR: 5'-CCGCTGCACTGTGAAGCTCT	206(bp)

G.P.: Generic Primers, SSP: Sequence specific primers.

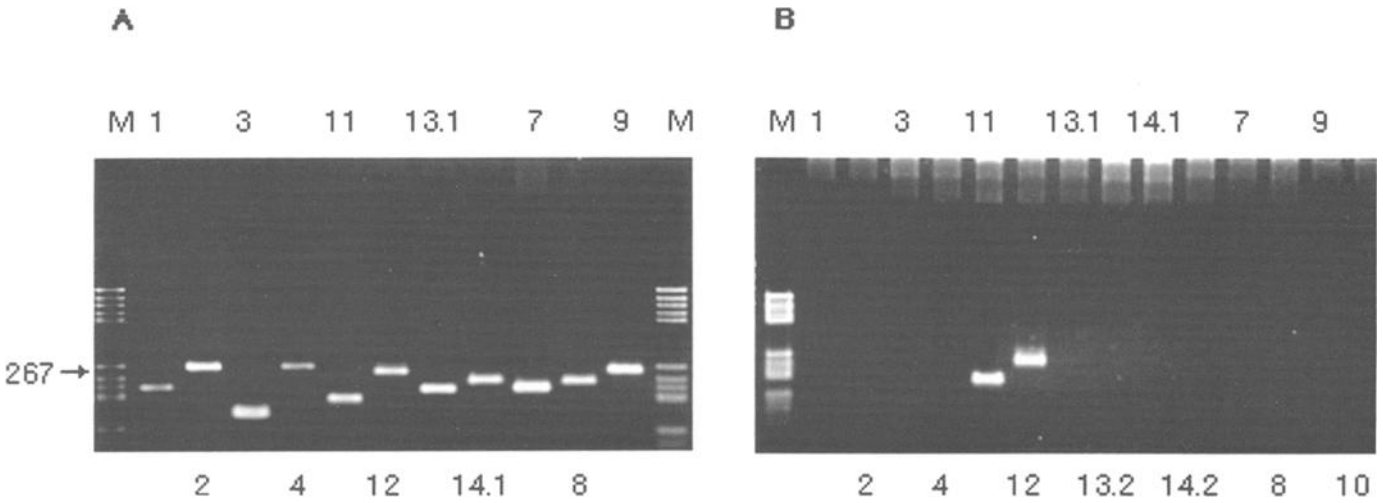

FIG. 1—*A): Illustration of the relative size of the PCR products obtained by the DR "low-resolution" PCR-SSP typing. DNAs used in each specific PCR reaction were extracted from the homozygous cell lines of the 10th International Histocompatibility Workshop (DR1:KAS116, DR2:KAS011, DR3:COX, DR4:LKT3, DR11:TISI, DR12:BM16, DR13.1:HHKB, DR14.1:TEM, DR7:PLH, DR8:BM9, DR9:DXB). Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. B): Illustration of typical HLA-DR "low-resolution" PCR-SSP typing using DNA sample obtained from whole blood cells of a healthy donor. Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Type: DR11/12 heterozygote, M: pBR322DNA digested by* HaeIII.

3′R12; 248bp, DR13.1: 5′R3568/3′R13.1; 203bp, DR14.1: 5′R3568/3′R14.1; 226bp, DR7: 5′R7/DRBR; 206bp, DR8: 5′R3568/3′R8; 225bp, DR9: 5′R9/DRBR; 256bp). This PCR-SSP method could identify polymorphisms corresponding to the serologically defined series DR1 to DR14. Figure 1B shows an example of SSP typing from one healthy person. DNA samples obtained from whole blood cells of randomly selected healthy individuals also could be typed correctly (data not shown).

In the Japanese population, DR2, DR4, and DR8 are common antigens. To yield much higher values of discrimination power

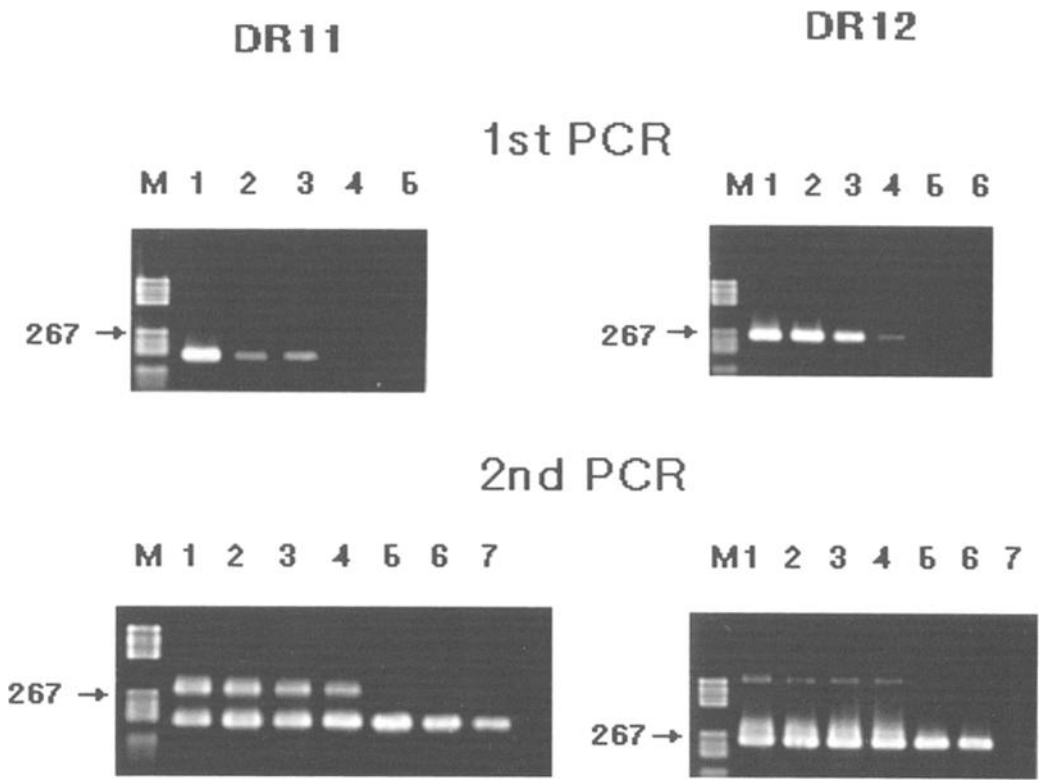

FIG. 2—*Checking the sensitivity of this method. Genomic DNA was obtained from DR11/DR12 heterozygous whole blood (Fig. 1B). After amplifying the DNA with generic primers (DRBF and DRBR), the second round PCR was performed by using 5′R3568 and 3′R11 (for 11 type) and 5′R12 and 3′R12 (for 12 type). Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. 1: 5 × 10^5 (pg), 2: 1 × 10^4 (pg), 3: 1 × 10^3 (pg), 4: 1 × 10^2 (pg), 5: 1 × 10^2 (pg), 6: 10 (pg), 7:1 (pg). M: pBR322DNA digested by* HaeIII.

and chance of exclusion, we used a high resolution analysis of the PCR-RFLP method (3) to distinguish the sub-alleles of these common DR2, DR4, and DR8 antigens. The PCR-RFLP method for DRB1 genotyping uses some informative restriction enzymes which have either a single cleavage site or alternatively no cleavage site in the amplified DNA region. The PCR amplified DNAs digested with restriction endonucleases (Fok I, Cfr13I, and HphI for DR2 group; SacII, AvaII, HinfI, HaeII, HphI and MnlI for DR4 group; AvaII, Fok I, Kpn I HaeII, Cfr 13I, SfaNI, SacII, BsaJI, ApaI, HphI, and RsaI for DR8 group) were subjected to electrophoresis. DRB1 genotypes were determined by checking whether the amplified DNAs are digested or not. This combined PCR-SSP and RFLP method is consequently able to discriminate DR1, DR2 (DRB1*1501 to *1602), DR3, DR4 (DRB1*0401 to *0411), DR11, DR12, DR13.1, DR13.2, DR14.1, DR14.2, DR7, DR8, (DRB1*0801 to *0804), DR9, and DR10. Of course, the ideal method would be successful typing of all 106 DRB1 alleles. But it is usually impossible to obtain a high degree of resolution from severely degraded or contaminated DNA, or the extremely small amounts of DNA extracted from forensic samples.

In this study, we tried to develop an easy-to-use, beneficial DNA typing method discriminating relatively high degree of HLA-DR polymorphisms in forensic practice. This assay system is easy to perform, and there is no need to use radioisotopes or many kinds of probes. In addition, this system yielded a discrimination power of 0.988 and 0.8404 for chance of exclusion, as calculated from each frequency in the Japanese population (Table 1). Accordingly, the method presented here is effective for use in forensic practice like microsatellite (13,14) and minisatellite (15,16) methods.

We examined the sensitivity of this method using DNA from fresh blood samples. Figure 2 shows the results obtained from genomic DNA of heterozygous samples (DR11 and DR12 shown in Fig. 1B). The PCR product of DR11 was amplified from 1 ng of diluted genomic DNA and visualized under UV after treatment with ethidium bromide using ordinary PCR with SSP. For DR12, the positive product was obtained at the concentration as low as 100 pg. To improve the sensitivity of the amplification reaction, we used nested or semi-nested PCR. Performing this reaction step, the generic primer pair (DRBF for 5′ primer just as for GH46, and DRBR for 3′ primer; see Table 1) was used in the first PCR, and SSPs were used for the second round of PCR. As shown in Fig. 2, the appropriate lengths of bands (178 bp for DR11, 248bp for DR12) were generated at 1 pg (DR11) and 10 pg (DR12) from the diluted genomic DNA in the second PCR. The extra bands

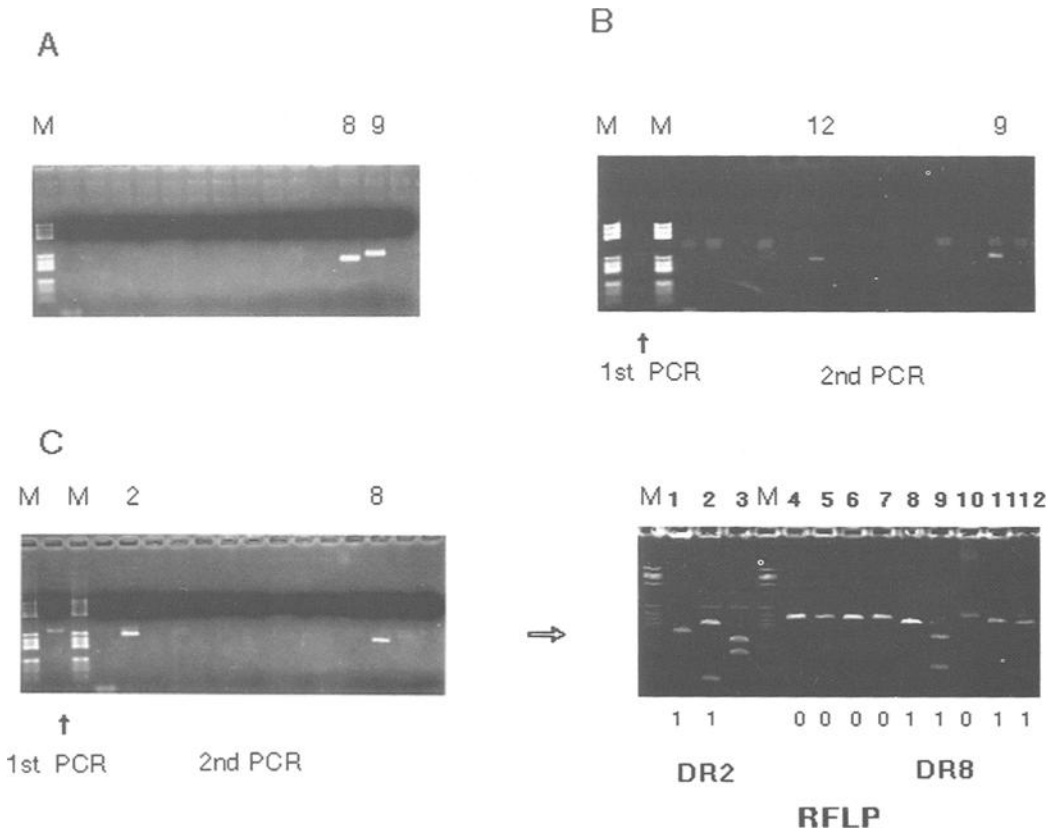

FIG. 3—*A) HLA-DR "low-resolution" PCR-SSP typing of a DNA sample extracted from the tooth. Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Type: DR8/DR9 heterozygote. M: pBR322DNA digested by* HaeIII. *B) HLA-DR DNA typing from bloodstains. The first PCR was done by DRBF and DRBR primers. DR12 and DR9 specific primers yielded positive bands on the lanes in the second PCR. Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. M: molecular size marker; pBR322DNA digested by* HaeIII. *Type: DR12/DR9 heterozygote, M: pBR322DNA digested by HaeIII. C) HLA-DR DNA typing from hairs. DR2 and DR8 lanes amplified by DR2 and DR8 specific primers showed positive fragments in the second round PCR (left figure). Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The DRB1 allele type was determined by the subsequent PCR-RFLP analysis (right figure). The PCR amplified DNAs digested with restriction enzymes were electrophoresed on a 12% polyacrylamide gel and stained with ethidium bromide. The sample was DRB1*1502/0804 heterozygote according to the cleavage patterns (1,1 for DR2 and 0,0,0,01,1,0,1,1 for DR8) by a series of restriction enzymes (3). M: pBR322DNA digested by* HaeIII. *The numbers 1 to 12 show restriction enzymes used in DRB1 allele typing. 1: Fok I, 2: Cfr13I, 3: HphI, 4: AvaII, 5: FokI, 6: KpnI, 7:* HaeIII, *8: Cfr13I, 9: SfaNI, 10: SacII, 11: BsaJI, 12: ApaI.*

TABLE 2—*DR typing by the PCR-SSP.*

S.D.	Primers Combination	Amplified Alleles (DRB1)	Frequencies (20)
DR1	5′-R1-DRBR	0101 ~ 0103	0.048
DR2	5′-R2-DRBR	1501 ~ 1602	0.154
DR3	5′-R3-3′R3	0301,0302	0.002
DR4	5′R4-DRBR	0401 ~ 0411	0.254
DR11(5)	5′R3568-3′R11	1101 ~ 1104	0.030
DR12(5)	5′R12-3′R12	1201,1202	0.066
DR13.1(6)	5′R3568-3′R13.1	1301,1302,1307	0.062
DR13.2(6)	5′R3568-3′R13.2	1303,1304,(0801,0803)	0.000
DR14.1(6)	5′R3568-3′R14.1	1401,1404,1405,1407	0.073
DR14.2(6)	5′R14.2-3′R14.2	1403,1406	0.029
DR7	5′R7-DRBR	0701,0702	0.008
DR8	5′R3568-3′R8	0801 ~ 0804,(1403)	0.126
DR9	5′R9-DRBR	0901	0.124
DR10	5′R10-DRBR	1001	0.006

S.D.: serologically defined antigen.

appeared around 600 bp in DR12 at second PCR are false and do not affect typing.

Figure 3A shows the results of the PCR products amplified with SSP from DNA sample extracted from the tooth. 50 ng of DNA (OD260/280 = 1.47) was added in each tube containing PCR mixture. In case we obtained enough quantity of DNA to determine HLA-DR types by PCR-SSP at first PCR, the second round PCR is not necessary.

As the total amounts of DNA obtained from the bloodstains and hairs were not enough to be subjected to amplification with SSPs, nested and semi-nested PCR methods were applied to determine DR type (Fig. 3B and 3C). As shown in Fig. 3B, the second-round PCR gave the positive fragments in the lanes using DR12 and DR9 specific primers. The nested and semi-nested PCR methods made it possible to conduct typing using small amounts of DNA. To type the DNA samples from hairs, we also used the nested and semi-nested PCR (Fig. 3C). The samples were typed as DR2 and DR8 heterozygotes. We then further analyzed the precise identity of the DRB1 alleles for DR2 and DR8 using the PCR-RFLP method (3). The sample was found to be a DRB1*1502/0804 heterozygote, based on cleavage patterns in the PCR-amplified products after treatment with restriction enzymes (Fig. 3C).

The usefulness of semi-nested PCR for forensic use was investigated for detecting HLA-DQA1 (17) and MCT118 (18). In this study, we evaluated the applicability of nested or semi-nested PCR for low resolution DR typing by applying the PCR-SSP method to extremely small amounts of DNA extracted from hairs and bloodstains. The results demonstrated these methods increased the sensitivity of the tests. Because Uchihi et al. (17) demonstrated that HLA-DQA1 DNA typing using the semi-nested PCR technique could detect contamination of single human sperm containing a single genome, in PCR procedure we should take all due precaution and avoid contamination from foreign DNA (19), especially in nested or semi-nested procedures so as not to obtain false positive results.

HLA DRB1 genes have the greatest variety of polymorphisms of the class II genes (DQA1, DQB1, DPB1). Higher degrees of polymorphisms are more useful in personal identification and paternity testing. However, determination of all the DRB1 alleles in one examination is difficult, time consuming and laborious. The typing system presented here has high efficiency with high discrimination (in terms of discrimination power and chance of exclusion) in the Japanese population. Furthermore, it makes typing from very small amounts of DNA possible. This method promises to be a useful tool for forensic investigations.

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