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**Original Article**

**INFLUENCE OF TEMPLATE DNA DEGRADATION  
ON THE GENOTYPING OF SNPs AND  
STR POLYMORPHISMS FROM FORENSIC  
MATERIALS BY PCR**

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**Abstract**

Detection of single nucleotide polymorphisms (SNPs) and short tandem repeat (STR) polymorphisms by PCR is widely used to analyze degraded DNAs in forensic science. The success of DNA analysis from human remains largely depends on the quality of the template DNA. We examined two SNPs (HLA-DQA1 and ABO) and two STR polymorphisms (VWA and CD4) by SSCP gel or denaturing gel electrophoresis, using two kinds of degraded DNA samples (165 teeth and blood stains contaminated with saliva) derived from the same person and investigated the influence of template DNA degradation on genotyping. As the degradation of DNA proceeds, unbalanced amplification of alleles occurred in the analysis of both SNPs and STRs, followed by allele drop, and further by loss of amplification. Non-target allelic products of STRs were amplified from highly degraded DNA samples; however, false allelic products of SNPs were not amplified from them. Amplification efficiency increased in proportion to the decrease of PCR target size, but reduction of the PCR target sizes also increased the chances of amplifying contaminating DNA, especially in highly degraded DNA specimens. The present results will help investigators to evaluate the genotyping of highly degraded DNA samples in forensic casework.

**Key words:** Forensic science—Degraded DNA—SNPs—STRs—  
Contamination—PCR

**INTRODUCTION**

DNA analysis has now become one of the major methodologies used to identify a human corpse. Because analysis of short tandem repeat (STR) polymorphisms has high discrimination power and the products

are relatively small, they are thought to be suitable for examining highly degraded DNAs. In particular, because commercially available multiplex STR typing kits have facilitated the genotyping of multiple loci at one time and their protocols have been validated, they are utilized in many countries to construct DNA

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This paper is a thesis submitted by H. Utsuno to the Graduate School of Tokyo Dental College.

databases<sup>4,5,24,25</sup>). As for single nucleotide polymorphisms (SNPs), the HLA-DQ $\alpha$  AmpliType typing system<sup>3,26</sup> and the AmpliType PM PCR amplification and typing kit are both commercially available and have been validated. Therefore, they have also been widely applied in forensic casework<sup>15</sup>). Although these kits and examination of other STR polymorphisms are very useful for forensic purposes, their genotyping is not always successful when DNA is highly degraded, and usually only the positive cases are reported<sup>8,12,16</sup>). The success of investigations largely depends on the degree of DNA degradation. Therefore, if we understand the characteristic amplification patterns in samples with different degrees of DNA degradation, the data will be helpful in evaluating the results of actual cases and elaborating counterplans in cases in which ambiguous amplification occurred. Thus, in this study, we investigated the influence of template DNA degradation on the genotyping of SNPs and STR polymorphisms by PCR.

We collected two kinds of samples derived from the same person; one was a tooth and the other was a blood stain samples contaminated with saliva. One group of tooth and blood stain samples was stored in a dry environment to minimize DNA degradation before DNA extraction, and the other group contained samples of many non-intact teeth stored in a damp environment for a various periods of time<sup>9</sup>) and tooth samples stored for long periods of time to create DNA samples with different degrees of degradation.

Two kinds of SNPs (HLA-DQA1 and ABO) and two kinds of STRs (VWA and CD4) were examined in these samples. These systems were chosen because they show variation in nucleotide sequence or length, the size of the repeat motif differ between the two STRs, VWA and CD4, and they are of forensic and anthropological interest.

## MATERIALS AND METHODS

### 1. Samples

Samples of teeth and cotton or gauze

patches used for hemostasis after extraction of teeth were obtained from 165 patients in a dental clinic. Informed consent was obtained from the patients, and this study was approved by the ethical committee of Tokyo Dental College. Cotton or gauze patches usually absorb blood and saliva (in this paper, we tentatively call the samples "blood stain samples"). Of these samples, 57 teeth and gauze patches were dried at room temperature immediately after extraction of teeth in order to minimize DNA degradation. DNA isolation from dried gauze patches were done in 1–2 weeks after collection of samples and that from teeth was performed within 1–7 months after tooth extraction. Isolated DNA samples were kept at 4°C. In contrast, the other 108 teeth and cotton patches were usually kept in a vinyl pouch and left at room temperature for 1–2 weeks in a damp environment after extraction of teeth and then were kept at room temperature. These tooth samples contained such things as highly decayed teeth, remaining roots, teeth with infected root canals, root-canal-treated teeth, or teeth broken in the course of tooth extraction. DNA isolation from cotton or gauze patches was done within the following two weeks. DNA isolation from 27 teeth was performed in 2–6 months after extraction of teeth, that from 34 teeth was done seven years after extraction of teeth, and that from 47 teeth was done nine years after extraction of teeth.

To isolate DNA from a tooth, the enamel layer was scraped with a dental instrument, and the remaining materials were crushed into powder using a "diamond mortar", a special device that crushes hard tissues, or Multi-beads Shocker (Yasui Co. Ltd.), followed by decalcification in 3 ml of 0.5 M EDTA for seven days. Although not all of the tooth powder dissolved in the EDTA, it was centrifuged, washed in TE twice, and re-suspended in TE, followed by proteinase K digestion. DNA was extracted twice with phenol/chloroform, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH7.6)<sup>13</sup>). Samples of cotton and gauze patches were washed in sterile physiological

saline twice, centrifuged, soaked in TE containing 1% sodium dodecyl sulfate (SDS), and digested with 0.1 mg/ml of proteinase K at 55°C overnight. The solution was transferred to another tube, and DNA was extracted as described above. The amount of DNA was quantitated spectrophotometrically using GeneQuant RNA/DNA calculator (Pharmacia Biotech).

## 2. PCR amplification

Segments of HLA-DQA1 were enzymatically amplified from genomic DNA using the following oligonucleotides as primers: primer HLA-1: 5'-GGTGTAACCTTGACCAGT-3', primer HLA-2: 5'-TTGGTAGCAGCGGTAGAGTT-3'<sup>10</sup>; these were constructed referring to the sequence database. This primer pair produces 227 bp fragments of the HLA-DQA1 locus (size of 0101 allele). Large and small segments of the ABO locus were amplified using the following oligonucleotides as primers: primer ABO-1: 5'-GCAGTAGGAAGGATGTCCTC-3', primer ABO-2: 5'-GACCTCAATGTCCACAGTCA-3'<sup>11</sup>, ABO-S(short)-1: 5'-AGTAGGAAGGATGTCCTCGT-3', and primer ABO-S-2: 5'-CTTCTTGATGGCAAACACAG-3'. The former pair produces 205 bp fragments, and the latter produces 138 bp fragments. The latter pair was constructed within the amplified region by ABO-1 and ABO-2 primers. The VWA locus was amplified as previously described<sup>17,20</sup>. The CD4 locus was amplified using primer pairs as previously described<sup>6</sup> and a new forward primer for CTTTT repeat strand; CD4-new-F: 5'-TCAAATAGGGGTACTTGTGT-3'. Each amplification of HLA-DQA1, ABO, VWA and CD4 was performed in a 30 µl mixture containing appropriate amounts of genomic DNA, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5–3 mM MgCl<sub>2</sub> (3 mM for HLA-DQA1, ABO and CD4, 2.75 mM for VWA, 1.5 mM for CD4 by Edwards *et al.*<sup>6</sup>), 0.01% gelatin, 200 µM dNTP, 400 nM of each primer, and 2.5 U of AmpliTaq Gold (Applied Biosystems).

The PCR conditions used for each system were 95°C for 10 min, followed by 30–35 cycles (30 cycles for VWA and 35 cycles for HLA-

DQA1, ABO and CD4) of denaturation at 95°C for 40–50 sec (40 sec for ABO and 50 sec for HLA-DQA1, CD4, and VWA), annealing and extension at 54–58°C (54°C for HLA-DQA1, 56°C for VWA, CD4, and 58°C for ABO) for 75–105 sec (75 sec for CD4, 90 sec for ABO and VWA, 105 sec for HLA-DQA1). The PCR cycling conditions used for ABO-S and CD4 by Edwards *et al.*<sup>6</sup> after 10 min denaturation at 95°C were: 35 cycles at 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec for ABO-S, and 30 cycles at 95°C for 30 sec, 55°C or 67°C for 30 sec, and 72°C for 1 min for CD4. After the last cycle, an extra step was performed at the final annealing and extension or extension for 10 min to extend the template completely.

## 3. Detection of polymorphisms

The PCR products of the HLA-DQA1 and ABO systems were analyzed by SSCP gel electrophoresis in a 17% polyacrylamide gel as described by Fujita and Kiyama<sup>7</sup> except that 0.5 × TBE and 1 × TBE were used for the gel and reservoir buffer, and a 16 × 36 cm gel with 0.4 cm thickness was used. Electrophoresis was performed at 55 V/cm constant voltage at 15°C for more than 7 hours. The PCR products of the VWA and CD4 loci were electrophoresed in 6% denaturing polyacrylamide gels, and those of the CD4 locus were further electrophoresed by an SSCP gel as described above except for using a 17% gel containing 5% glycerin. All the products were visualized by silver staining.

## 4. Sequencing analysis

To determine the DNA sequence of an allelic product, the amplified DNA fragment was eluted from silver stained gels using the “crush and soak” method<sup>21</sup>, re-amplified by PCR, and directly sequenced using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence analysis was performed on an ABI 310 DNA Sequencer.

## RESULTS

### 1. Typical electrophoretogram of the HLA-DQA1, ABO, and the CD4 loci by SSCP gel electrophoresis

SSCP gel electrophoresis usually produces a complicated electrophoretogram. In order to demonstrate reliability for typing, we describe typical SSCP gel electrophoretograms of the HLA-DQA1, ABO, and CD4 loci using DNA samples isolated from blood by ordinary protocol.

In the analysis of the HLA-DQA1 locus, the seven common allelic products (0101, 0102, 0103, 0301, 0401, 0501, 0601) and a new one (0301\*) having a pair of bands with similar intensity could be differentiated (Fig. 1A). Because migration of the allelic products of 0401 and 0601 and those of 0101 and 0102 differed slightly from each other, respectively, it was necessary to load known marker samples side by side on a gel to type these alleles. Although the new allelic product found in this study migrated quite differently from other allelic products in an SSCP gel (Fig. 1A, lane 4), the sequence differed in only one nucleotide from that of the allele 0301 (Fig. 1A, lanes 5–7). Because no identical sequence could be found in the human genome database, we tentatively designated this allele as 0301\* (Fig. 1A, lane 4). When the amplification efficiency was low, especially in the typing of degraded DNAs, it was necessary to load larger amounts of amplicons on a gel. Changing the ratio of the amplified solution to sample loading formamide solution resulted in different intensities of major and minor bands on an SSCP gel of the HLA-DQA1 system (not shown). In order to maintain the intensity of major bands for typing the HLA-DQA1 system, the volume of sample loading solution should be more than twice as much as that of the PCR solution.

In the analysis of the ABO locus, we amplified a 205 bp fragment using a primer pair, ABO-1 and ABO-2, and further amplified a 138 bp fragment using a primer pair, ABO-S-1 and ABO-S-2, for typing some of the samples. SSCP gel electrophoresis clearly

discriminated four kinds of alleles (A, B, OA, and OG)<sup>1)</sup> having a pair of bands with similar intensities in both systems (Figs. 1B and 1C).

We amplified the CD4 locus using primers reported by Edwards *et al.*<sup>6)</sup> and a new forward primer constructed in this study. Because there is a SNP at the 3' end of their forward primer<sup>28)</sup>, the new forward primer was constructed from a position 26 bp upstream of their CTTTT primer. Although ordinary typing was performed by denaturing the gel, SSCP gel electrophoresis was used to discriminate a SNP at the 3' end. By SSCP gel electrophoresis, two types of allele 4, 4A and 4C, could be further differentiated (Fig. 1D). The electrophoretogram of alleles 5 to 10 was somewhat complicated in an SSCP gel because of heteroduplex formation. However, they could be identified in comparison with the genotype obtained by denaturing polyacrylamide gel electrophoresis. Only one type was observed in alleles 5, 6, 8, 9, and 10.

### 2. Typing of the HLA-DQA1, ABO, VWA and CD4 loci using tooth and blood stain samples dried immediately after extraction

Segments of HLA-DQA1, ABO, VWA and CD4 were amplified using DNA obtained from 57 blood stain and tooth samples dried immediately after extraction of teeth. PCR amplification was first performed using 10 ng of genomic DNA, and when no products or faint bands were obtained, 20 ng of DNA was further used for a second amplification. Amplification products of all four loci were obtained from every sample, and allelic products were clearly discernible on an SSCP or a denaturing polyacrylamide gel. The genotypes of two SNPs, HLA-DQA1 and ABO, obtained from tooth and blood stain samples matched each other, and no sample was found with different band intensities in heterozygotes. In the typing of two STRs, VWA and CD4, genotypes obtained from tooth and blood stain samples matched each other, and no sample was found with different band intensities in heterozygotes in these cases, either. Shutter bands caused by slippage artifacts were more pronounced in the VWA locus

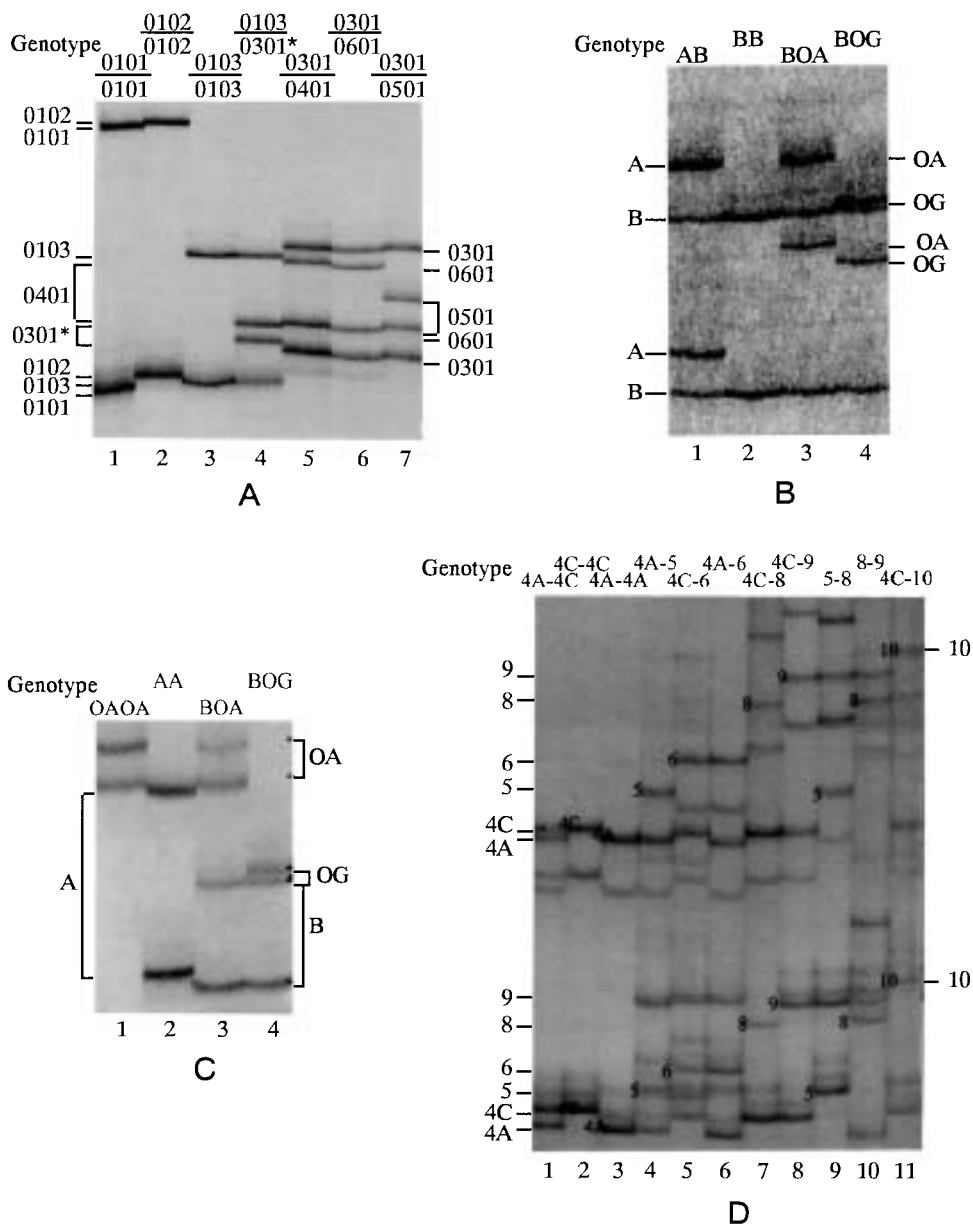


Fig. 1 Typical electrophoretogram of the HLA-DQA1, ABO, and CD4 loci by SSCP gel electrophoresis. A: SSCP gel electrophoresis of the HLA-DQA1 locus amplified using blood samples from seven individuals. Migration positions of the six allelic products and a new one (0301\*) are indicated on both sides of the gel, and the genotype of each sample is shown on the top. B: SSCP gel electrophoresis of the ABO locus amplified using blood samples from four individuals. Migration positions of the four allelic products are indicated on both sides of the gel, and the genotype of each sample is shown on the top. C: SSCP gel electrophoresis of ABO-S amplified using blood samples from four individuals. Migration positions of the four allelic products are indicated on both sides of the gel, and the genotype of each sample is shown on the top. Each allele could be determined by a pair of bands. D: SSCP gel electrophoresis of the CD4 locus amplified using blood samples from 11 individuals. Because of hetero duplex formation, three kinds of allelic bands were observed. Migration positions of two of them for 4A, 4C, 5, 6, 8, 9, and 10 are indicated on both sides of the gel, and the genotype of each sample is shown on the top.

than in the CD4 locus (not shown). However, the intensities of these shutter bands did not differ from those observed when blood samples were used as templates. Thus, we did not have any difficulty in the typing of these four loci from all of the samples.

### 3. Typing of the HLA-DQA1, ABO, VWA and CD4 loci using tooth and blood stain samples with different degrees of degradation

Segments of HLA-DQA1, ABO, VWA, and CD4 were amplified using DNA from 108 blood stain and tooth samples. These samples were stored in a damp environment for at least one to two weeks after collection, and some of the tooth samples, including many non-intact teeth, were stored for much longer periods of time, as described in Materials and Methods. Therefore, we expected to obtain DNA samples with different degrees of degradation. PCR amplification was first performed using 10 ng of genomic DNA, and when no products or faint bands were obtained, 20 ng of DNA was used for a second amplification. When amplification products were still not obtained or faint bands were obtained, re-amplification from the first PCR mixture (dual PCR) was carried out. In addition, when faint bands or no bands were obtained after dual PCR at the ABO locus, the smaller segment, ABO-S, was amplified. Thus, amplification efficiency depending on the difference of PCR target size on the same locus could be compared.

Based on the process of genotyping and the issue of amplification, we classified these 108 blood stain and 99 tooth samples into three

groups. Because nine of the DNA samples from teeth were completely consumed during the study, their data were not included in the groupings. As shown in Table 1, 70 blood stain samples (64.8%) and 73 tooth samples (73.7%) could be genotyped at all of the four loci using 10 or 20 ng genomic DNA. These samples were classified as group I in this study. For 8 blood stain samples and 11 tooth samples, dual PCR had to be carried out to type some of the four loci; these were classified as group II. The small segments of ABO (ABO-S) were also amplified from the samples of group II. From 30 blood stain samples and 15 tooth samples, PCR products of some of the four loci, including ABO-S, could not be obtained after dual PCR. These samples were regarded as highly degraded DNA samples in the following study and were classified as group III. Table 2 shows the results, whether amplification products were obtained or not obtained, in the target region of each system in the samples of group III. The order of amplified product sizes was HLA-DQA1 (227 bp), ABO (205 bp), VWA (138–162 bp), ABO-S (138 bp), and CD4 (112–142 bp). In 21 blood stain samples and 13 tooth samples in which prominent amplification products could not be obtained in the original PCR of the ABO loci, the smaller PCR fragment, ABO-S, could be amplified from 15 and 8 samples, respectively (Table 2). The total number of samples from which amplification products were not obtained or obtained out of the target region increased in proportion to the increase in the PCR target size.

Unlike the samples dried immediately

Table 1 Distribution of number of blood stain and tooth samples with different degree of degradation classified into three groups

	Group I	Group II	Group III	Total
Blood stain	70 (64.8%)	8 (7.4%)	30 (27.8%)	108
Tooth	73 (73.7%)	11 (11.1%)	15 (15.2%)	99

Table 2 Combination of the results showing whether amplification products were or were not obtained in the target regions among the samples classified in Group III

Blood stain samples						
	HLA-DQA1	ABO	ABO-S	VWA	CD4	No. observed
	–	+	+	+	+	9
	–	–	+	+	+	11
	–	–	+	–	+	2
	–	–	+	–	–	2
	–	–	–	–	+	3
	–	–	–	–	–	3
Total no. of —	30	21	6	10	5	
Tooth samples						
	HLA-DQA1	ABO	ABO-S	VWA	CD4	No. observed
	–	+	+	+	+	2
	–	–	+	+	+	4
	–	–	+	–	+	3
	–	–	–	+	+	2
	–	–	+	–	–	1
	–	–	–	–	+	2
	–	–	–	–	–	1
Total no. of —	15	13	5	7	2	

+ : amplification products were obtained in the target region.

– : amplification products were not obtained or obtained out of the target region.

after sampling, we encountered misleading amplification in samples stored in a damp environment for both SNPs and STRs (Table 3; Figs. 2 and 3).

In the Group I samples, unbalanced amplification of heterozygotic alleles occurred; we defined this pattern as allelic imbalance in this paper. It occurred in both SNP and STR analysis of DNA samples derived from blood stains and teeth (Table 3; Fig. 2A, lane 3; Fig. 3A, lane 3; Fig. 3B, lanes 3 and 6). However, because we did not find inconsistencies in SNP and STR genotypes between tooth and blood stain samples in Group I, we thought that the faint bands in heterozygotes were the products of target sequences.

In the Group II samples, allelic imbalance and allele drop were frequently observed (Table 3; Fig. 2A, lanes 6 and 9; Fig. 2B, lanes 2 and 5; Fig. 3A, lane 6; Fig. 3B, lane 9). In this group, some of the results were obtained using dual PCR. When faint bands were

detected in the first PCR, they became intense by dual PCR. However when no band was detected in the first PCR, dual PCR usually did not dramatically improve amplification. Other misleading amplifications encountered in group II (Table 3) were 1) insufficient amplification after dual PCR (HLA-DQA1 and ABO), and 2) amplification of a false allele and an additional allele-like band out of the allelic ladder (VWA) (Fig. 3A, lanes 8 and 9). In the first example, small-sized PCR products, ABO-S, VWAs and CD4, were well amplified using 10 ng of template DNA from a blood stain sample; however, larger-sized products, HLA-DQA1 and ABO, were faint even after dual PCR. In the second example, a false allele and an allele-like band out of the allelic ladder were amplified by dual PCR. In this example, other loci were also genotyped by dual PCR. Thus, these samples may also be highly degraded, even though they were classified into Group II.



Table 3 Misleading amplification observed in typing of the samples with different degree of degradation

	Blood Stain				Tooth			
	No. amplified	Allelic imbalance	Allele drop	Others	No. amplified	Allelic imbalance	Allele drop	Others
<b>Group I</b>								
HLA	70	1			73	1		
ABO	70	3			73	1		
VWA	70	1			73	1		
CD4	70	1			73	1		
<b>Group II</b>								
HLA	8		1		11	1	3	1
ABO	8				11	1	1	1
ABO-S	8				11		1	
VWA	8	3			11	5	1	1
CD4	8	1			11		1	
<b>Group III</b>								
HLA	0				0			1*
ABO	9			1	2			1
ABO-S	24				10	1		1
VWA	20	3	1	2	8			
CD4	25	3		1	13	2		3

No. amplified: Number of samples where amplified products were obtained.

Allelic imbalance: Unbalanced amplification of heterozygotic alleles.

Others: Problems other than allelic imbalance and allele drop.

\*: Unidentifiable bands were amplified in the target region, although target products were not amplified.

In the Group III samples, negative amplification was the most frequently observed (Table 2). Other misleading amplifications (Table 3) were 1) allelic imbalance (ABO-S, VWA, and CD4) (Fig. 2C, lane 2; Fig. 3A, lane 12), 2) allele drop (VWA) (Fig. 3A, lane 14), 3) amplification of different alleles from the same sample in different PCR (VWA) (Fig. 3A, lanes 17 and 18), 4) ladder pattern (VWA) (Fig. 3A, lane 21), 5) positive and negative amplification from the same sample in different PCRs (once in ABO and twice in CD4), 6) amplification of unidentifiable bands in the target region (HLA-DQA1 and ABO-S) (Fig. 2A, lane 12; Fig. 2C, lane 4), 7) increase in backgrounds (twice in CD4) (Fig. 3B, lane 12), and 8) increased sensitivity for contamination (ABO-S). Because the template DNA of this group may be highly degraded, misleading amplification observed in the samples of Group III may be caused mainly by template DNA degradation. When contamination with foreign DNA occurred in the PCR mixture,

it was well amplified in the samples of this group. The amplification efficiency of small loci is generally higher than that of larger loci. Contaminated DNA mixed up with a PCR mixture was more frequently amplified in the analysis of small loci, especially in that of ABO-S. Our results show that an increase in the amplification efficiency of target DNA also increases the amplification efficiency of contaminating DNA.

## DISCUSSION

Degradation of template DNA affects DNA typing. Success or failure of DNA typing mainly depends on the degree of template DNA degradation. Therefore, it is important to understand the influence of template DNA degradation on DNA analysis by PCR. In this study, we collected large numbers of two kinds of samples, tooth and blood stain samples, from the same individuals, and isolated DNAs

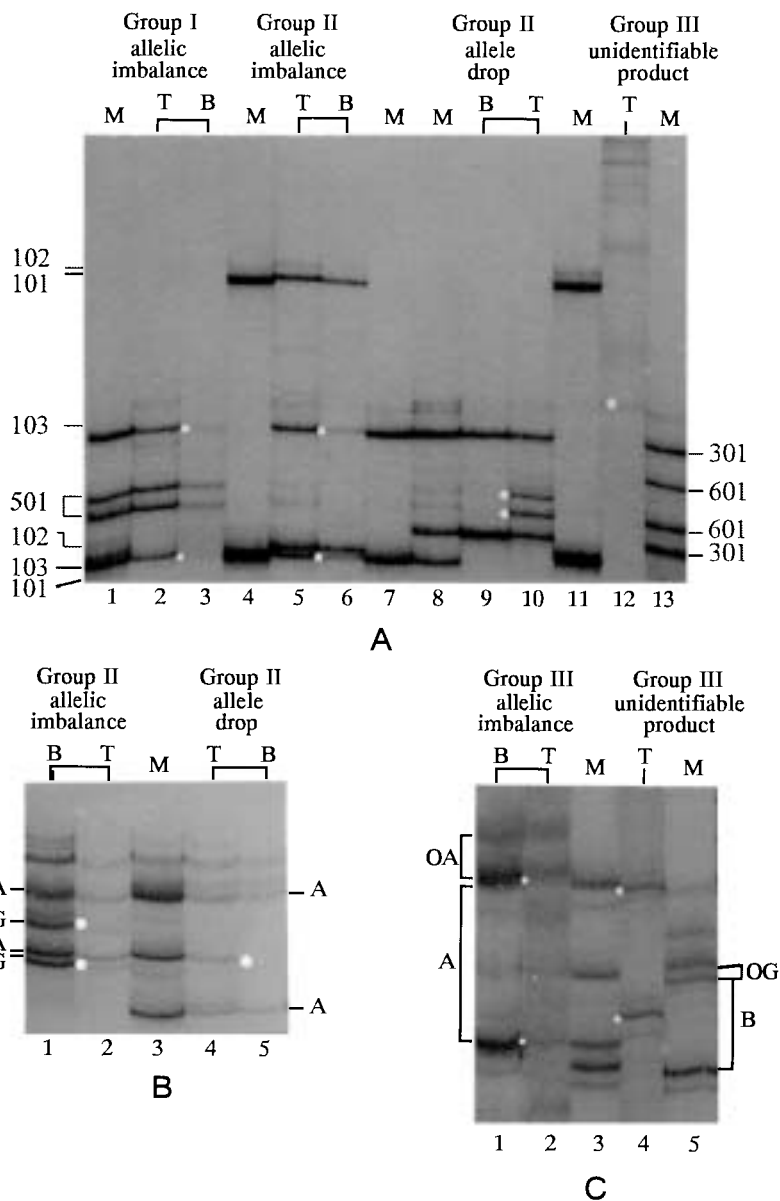


Fig. 2 Electrophoretogram of the HLA-DQA1 and ABO loci obtained from tooth and blood stain samples showing misleading amplification. Migration positions of the allelic products are indicated on both sides of the gels. Sources of samples, classification of the group of samples, and phenomena observed are shown on the top. T shows a tooth sample, B shows a blood sample, and M shows a known marker sample. A: Electrophoretogram of the HLA-DQA1 locus. Channels 2 and 3, 5 and 6, 9 and 10 are derived from the same individuals, respectively. Channel 1: 0103/0501; Channel 2: 0103/0501; Channel 3: 0103/0501, white dots on the left show weak amplification of the allele 0103; Channel 4: 0101/0101; Channel 5: 0102/0103; Channel 6: 0102/0103, white dots on the left show weak amplification of the allele 0103; Channel 7: 0103/0103; Channel 8: 0103/0301; Channel 9: the sample is of 0301/0501 type but lacks bands for the allele 0501 (shown by dots on the right); Channel 10: 0301/0501; Channel 11: 0101/0101; Channel 12: a white dot on the left shows an unidentifiable band which does not correspond to any alleles; Channel 13: 0301/0601. B: Electrophoretogram of the HLA-DQA1 locus. Channels 1 and 2, and 4 and 5 are derived from the same individuals, respectively. Channel 1: OAOG; Channel 2: OAOG, white dots on the left show weak amplification of the allele OG; Channel 3: AOA; Channel 4: AOA; Channel 5: the sample is of AOA type but lacks a band for the allele OA (shown by a dot on the left). C: Electrophoretogram of the ABO-S fragment. Channels 1 and 2 are derived from the same individual. Channel 1: AOA; Channel 2: AOA, white dots on the left show weak amplification of the allele A; Channel 3: AB; Channel 4: white dots on the left show unidentifiable bands which do not correspond to any alleles; Channel 5: BOG.

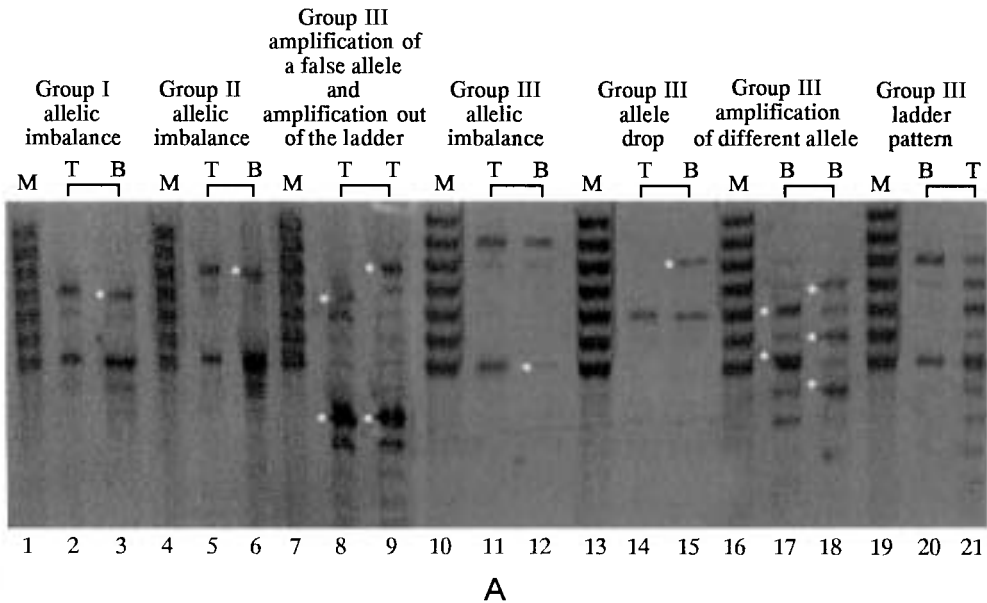
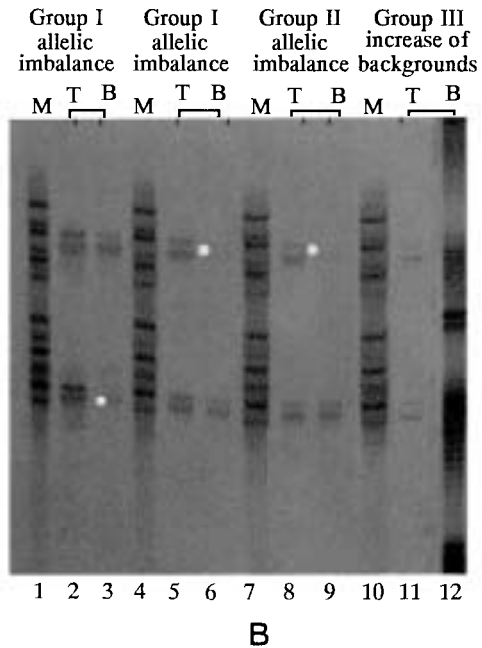


Fig. 3 Electrophoretogram of the VWA and CD4 loci obtained from tooth and blood stain samples showing misleading amplification. Source of samples, classifications of the group of samples, and phenomena observed are shown on the top. T shows a tooth sample, B shows a blood sample, and M shows an allelic ladder. A: Electrophoretogram of the VWA. Allelic ladder contains allelic products from 14 to 20. Channels 2 and 3, 5 and 6, 11 and 12, 14 and 15, 20 and 21 are derived from the same individuals, respectively. Channels 8 and 9, and 17 and 18 are amplification from the same samples, respectively. Channel 2: 14–17; Channel 3: 14–17, a white dot on the left shows weak amplification of the allele 17; Channel 5: 14–18; Channel 6: 14–18, a white dot on the left shows weak amplification of the allele 18; Channel 8 and 9: the upper white dots show different allelic products (alleles 17 and 18) amplified from the same sample, and the lower white dots show allele-like bands out of the allelic ladder; Channel 11: 14–19; Channel 12: 14–19, a white dot on the left shows weak amplification of the allele 14; Channel 14: 16–18, a white dot on the right shows weak amplification of the allele 18; Channel 15: 16–18; Channels 17 and 18: white dots on the left show different allelic products amplified from the same sample; Channel 20: 14–18; Channel 21: many bands with similar intensity were amplified. B: Electrophoretogram of the CD4 locus. Allelic ladder contains allelic products from 4 to 10 except 7. Channels 2 and 3, 5 and 6, 8 and 9, 11 and 12 are derived from the same individuals, respectively. Channel 2: 4–9; Channel 3: 4–9, a white dot on the left shows weak amplification of the allele 4; Channel 5: 4–9; Channel 6: 4–9, a white dot on the left shows weak amplification of the allele 9; Channel 8: 4–9; Channel 9: 4–9, a white dot on the left shows weak amplification of the allele 9; Channel 11: 4–9; Channel 12: allelic products could not be assigned because of high backgrounds.



from the samples stored under different conditions, expecting to be able to obtain template DNAs with different degrees of degradation. In order to prepare degraded DNA samples, we kept 108 samples in a damp environment for one to two weeks. This condition makes blood stain samples degrade very rapidly. However, it was not obvious to what extent this condition made tooth samples degrade. Although the long storage of these samples might also influence the degradation of DNA, we could not elucidate a clear correlation between the degree of degradation and the period of storage. The reason is that the samples we collected included non-intact teeth and those infected from the beginning, so the condition of the teeth might largely have affected the degree of degradation of the samples. Because the main purposes of this study were to examine the success or failure of typing SNPs and STR polymorphisms and to clarify problems in failure of PCR amplification using samples with different degree of degradation irrespective of storage condition, we did not consider the storage periods of samples and the conditions for selection of samples to be critical issues.

The Cetus Amplitype HLA-DQA forensic DNA amplification kit has been widely used in the forensic field<sup>3,26</sup>. Allele specific PCR and PCR-RFLP (restriction fragment length polymorphisms) methods have also been used to type the HLA-DQA1 and ABO systems<sup>11,14,19,27</sup>. We used SSCP analysis<sup>1,7</sup> to type two SNPs, HLA-DQA1 and ABO, because it was possible to amplify heterozygotic alleles using the same primers and to detect products directly by silver staining, thereby allowing us to eliminate further steps such as hybridization and enzyme digestion and making conditions suitable to compare differences of amplification efficiency between the alleles amplified under the same condition.

PCR products were well amplified from the samples dried immediately after collection. Because prominent PCR products were obtained from all of the samples and no inconsistency of genotypes between blood stain and tooth samples were observed, typing

from these samples must be reliable. In the samples of Group I with different degrees of DNA degradation, amplification products of the four loci were obtained in the first PCR, including the samples showing allelic imbalance. Although allelic imbalance was observed, typing of these samples may also be reliable, because no inconsistency of genotypes were observed between blood stain and tooth samples. In contrast, allele drop resulting in false genotyping was a frequently observed problem in Group II, in which dual PCR was necessary to type some of the four loci. Allelic imbalance was also frequent in Group II. In Group III, allelic imbalance and allele drop were also observed, but failure of amplification was the most frequent problem. From these results, it can be inferred that, as degradation of template DNA proceeds, three phenomena occurs in this order: allelic imbalance, allele drop, and negative amplification. Although negative or weak amplification could also be due to PCR inhibition, we did not do further experiments to determine whether the loss of amplification or weak amplification was due to degradation or PCR inhibition. However, we presume that they were due to degradation in this study, because complete loss of amplification in the PCR of the present five systems (Table 2) was found in only four DNA samples extracted under the similar conditions. Imbalance of STR allele in the amplification using degraded DNA is also reported by others<sup>5,26</sup>. Allele drop in the amplification of STRs from highly degraded DNA has been reported by many researchers<sup>2,5,18,22,23,25</sup>. It can be speculated that, when allelic imbalance was observed, the size of amplifiable template DNA might be close to the PCR product size. Thus the possibility of misleading amplification shown in this paper should be considered for typing other polymorphisms in such samples, because allelic imbalance is the first sign of the progression of DNA degradation.

When allelic imbalance was observed in STR typing, a larger allele was usually fainter than a smaller one. This result coincides with another result that the amplification efficiency of a smaller locus was higher than that of a

larger one. However, we had two examples in Group III that allele 19 (158 bp) was better amplified than allele 14 (138 bp) in the VWA system (Fig. 3A, lane 12) and that allele 9 (133 bp) was better amplified than allele 4C (108 bp) in the CD4 system (Fig. 3B, lane 3). A difference in the degree of degradation in the template DNA between the two allelic regions in the same locus might be one of the reasons for this phenomenon, although other reasons might be speculated.

Amplification of a false allele was found in two samples of VWA (Fig. 3A, lanes 8 and 9, lanes 17 and 18). One belongs to Group II, whose typing of all of the four loci and ABO-S had been done by dual PCR. The other belongs to Group III, and its HLA-DQA1 and ABO loci had not been amplified (not shown). Thus, both samples may be highly degraded. If the target band was not well amplified in the first PCR due to degradation of DNA, non-target products caused by slippage, insufficient amplification, or contamination might have been emphasized by dual PCR. The ladder pattern observed in the sample of Group III (Fig. 3A, lane 21) may be caused by a similar reason. Slippage artifacts occur more frequently in the VWA locus than in the CD4 locus, because the repeat unit is smaller in VWA (4 bp) than in CD4 (5 bp). This is one of the reasons why a false allele was more frequently observed in the analysis of the VWA locus.

Unidentifiable bands amplified in the target regions of the ABO-S and the HLA-DQA1 loci were found in the same sample (Fig. 2A, lane 12, Fig. 2C, lane 4) whose patterns of VWA and CD4, determined by dual PCR, were not different from those of blood samples. Although we could not determine the nucleotide sequences of these unidentifiable bands, other regions with sequences similar to those primers might have been amplified.

As shown in this paper, misleading amplification such as unbalanced amplification of heterozygotic alleles, amplification of a false allele, and ladder patterns occurred in STR typing of highly degraded DNA. It is difficult to elucidate whether they occurred because

of degradation of DNA or because of contamination with foreign DNA. In contrast, it was sometimes possible to find contamination with foreign DNA in SNP typing when a third allele was found. This occurred in our experiments with ABO-S amplification because of our technical error. Allelic bands were amplified in the blank PCR. Needless to say, they disappeared in the PCR using new reagents. However, contaminating allelic bands were not found in all of the samples amplified using the same PCR mixture and were more frequently detected in the PCR of highly degraded DNA samples. Although we could not determine whether all of the samples we isolated were contaminated with foreign DNA or not, there were a few samples showing an additional faint band corresponding to the third allele in spite of negative amplification in the blank PCR. Because we isolated DNAs under the same conditions, some of other samples, such as those dried immediately after collection, could also have been contaminated with foreign DNA to a small extent. However, we could not confirm any contamination with foreign DNA in these samples. We presume that contaminated DNA bands might be emphasized when degradation of template DNA becomes severe. In addition, contaminated target bands were more frequently detected in the PCR of ABO-S than in that of ABO or HLA-DQA1 with a larger target size. Reduction of target size significantly increases amplification efficiency from highly degraded DNA samples, as shown in this paper and by others<sup>29)</sup>, but also increases the detection of contaminating DNA bands, depending on the amount of contaminating template DNA. Trials to increase PCR amplification efficiency must always be associated with the development of trials to prevent contamination.

In this study, we prepared DNA samples with different degrees of degradation and investigated the influence on PCR amplification of SNPs and STR polymorphisms. As the degradation of DNA proceeded, the reliability of PCR amplification dropped. Reduction of PCR target size is one of the ways to overcome this problem. However it also increases the

sensitivity to contamination, especially in the analysis of degraded DNA. Unbalanced amplification of heterozygotic alleles is a first sign of the progress of DNA degradation. When other misleading PCR amplification patterns shown in this paper are observed, we should elaborate counterplans in consideration of the degree of degradation of amplifiable DNA.

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