TECHNICAL NOTE

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Purification of Forensic Specimens for the Polymerase Chain Reaction (PCR) Analysis

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ABSTRACT: Purification methods of deoxyribonucleic acid (DNA) from degraded and contaminated forensic samples were investigated for polymerase chain reaction (PCR) analysis. DNA extracted from putrefied tissue or bloodstains sometimes contained the copurified contaminant, that was identified as the porphyrin compound (hematin). When contaminated but less degraded DNA was analyzed by PCR, it was necessary to eliminate the impurity by anion exchange column chromatography or chelating resin preparation, and ultrafiltration using Centricon microconcentrators. When highly degraded DNA was analyzed, trace amounts of high molecular weight DNA was recovered by electroelution method, and then further purified by both column chromatography and ultrafiltration. From thus purified samples, the amelogenin gene for sex determination could be amplified by dual PCR technique.

KEYWORDS: pathology and biology, deoxyribonucleic acid (DNA), polymerase chain reaction (PCR), postmortem degradation, amelogenin gene, porphyrin compound

Analysis of deoxyribonucleic acid (DNA) has been applied to forensic practices such as individualization, parentage testing, sex determination, and species identification. DNA in forensic specimens is, however, often present in trace amounts and/or contaminated [*1–5*]. Polymerase chain reaction (PCR) is powerful for DNA analysis of such specimens, since, theoretically, it can be used to detect genetic material from as little as single cell of sample tissue [*6*]. Forensic PCR analysis has thus been explored, including sex determination by amplification of sex chromosomal genes, such as Y chromosomal repetitive DYZ1 sequence [*7,8*], centromeric α-satellite repeats of X and Y chromosomes [*9–11*], and X-Y homologous amelogenin gene [*8,12*].

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Generally, amplification of multiple-copy genes is quite sensitive. For example, 1024 base pair (bp)-B fragment of DYZ1 sequence has been effectively amplified from 0.24 ng of template DNA and even from putrefied tissue samples [*8*]. When the X-Y homologous amelogenin gene was amplified using a set of primers, 977 bp X and 788 bp Y counterparts were detected at the same time [*8,13*]. This difference in the size was attributed to that of an intron [*14*], and the X-fragment acted as an internal control that validated the analytical procedures. In contrast to DYZ1 sequence, PCR amplification of the single-copy amelogenin gene required at least 250 ng/reaction of template DNA [*8*]. Then, dual PCR technique was applied to increase the sensitivity [*12,15*], by which the target gene is amplified with a set of primers, and then reamplified using nested primers [*16,17*]. This technique also improves the PCR specificity, since the nested primers anneal to the sequence internal to the two outer primers so as to identify the subset of amplification products that corresponds to the target fragment [*17*]. By this technique, low-sensitive amelogenin gene was detectable from as little as 0.005 ng of template DNA [*12*]. However, the gene could not be amplified from putrefied tissue samples even by dual PCR. These facts suggested that the PCR analysis of such low-sensitivity single-copy gene was easily affected by the contamination and/or degradation of sample DNA.

Thus, forensic scientists have two choices in the PCR analysis of such impure samples. The first is to selectively analyze multiple-copy genes such as the repetitive sequences [*7–11*] and mitochondrial DNA [*18,19*]. These genes may be easily detected from trace and contaminated samples [*8,19*]. However, PCR amplification of some single copy genes, such as amelogenin [*8,15*] and HLA-DQα genes [*5,17,20–23*], may be required in some forensic cases. Then, the second choice, to establish purification protocols of impure specimens for forensic PCR analysis is also quite important.

Materials and Methods

Preparation of Impure DNA Samples and Identification of the Contaminant

Pure human genomic DNA was extracted by Proteinase K and phenol/chloroform treatment from male and female fresh peripheral leukocytes [*24*]. Degraded and contaminated samples were isolated from ventricular blood, quadriceps femoris muscle, brain, and liver of an old woman, who died in a bed in late summer and was found 7 days later. Other DNA samples were extracted from dried bloodstain and adipose tissue, which were found on the surface of an electric-light pole at a traffic accident scene and thought to be the blood from a woman killed in the accident. Experimental bloodstains were also prepared by spotting 10 μL fresh blood (female material) onto cotton cloth. DNA in these extracts was quantitated by optical density at 260 nm (OD_{260}) minus OD_{310}, and their purity was checked by the ratio OD_{260}/OD_{280} [*25*]. The spectrum of impure sample from the liver tissue was also examined using Hitachi 220A double beam spectrophotometer. The DNA sample from liver was purified for PCR by column chromatography or electroelution method, and/or ultrafiltration as described below.

The impurity in the sample was identified by pyridine hemochromogen analysis [*26*]. Briefly, the dialysate of Centricon® 100 (Amicon, Beverly, MA) was lyophilized, and dissolved in 0.5 mL pyridine. After the addition of 0.5 mL 0.2 N potassium hydroxide, the impurity was oxidized by a small drop of 0.05 M potassium ferricyanide, and the absorbance at 556 nm of pyridine *ferri*chromogen was determined. Following the reduction by sodium hydrosulfite, the spectrum of pyridine *ferro*chromogen was recorded. The content of heme *b* was calculated by the equation of

$$\text{heme } b\ (\mu\text{M}) = \frac{\Delta OD_{556}\ (\text{reduced form-oxidized form}) \times 1000}{30} \quad [26].$$

Sample Concentration and Removal of Decomposed, Low Molecular Weight (LMW) DNA by Ultrafiltration

The DNA sample from the putrefied liver was diluted in 2.0 mL of TE buffer [consisted of 10 mM tris(hydroxymethyl)aminomethane (Tris)/hydrochloride (HCl) and 1 mM ethylenediaminetetraacetic acid (EDTA), at pH 8.0] and applied to the upper compartment of Centricon® 30 or 100 microconcentrator (Amicon). It was then centrifuged at 3000G or 1000G for 30 min at 4°C, respectively. The remaining solution in the upper compartment was recovered, and then, ethanol precipitation was performed [*25*] to dissolve DNA in appropriate volume of TE buffer.

Purification of High Molecular Weight (HMW) Genomic DNA by Anion Exchange Column Chromatography

The A.S.A.P.™ Genomic DNA Isolation Kit (Boehringer Mannheim, Mannheim, Germany) was used to remove the impurities in the liver sample, according to the supplier's instruction. Briefly, the sample solution was applied to the anion exchange column matrix after the addition of 4 mL of column wash buffer in the kit. The column matrix was washed by 6 mL of wash buffer. After priming the column with 0.5 mL of elution buffer in the kit, DNA was recovered using 2 mL of elution buffer. The eluted sample was then concentrated by ethanol precipitation, or by ultrafiltration and ethanol precipitation as described above.

Recovery of HMW DNA by Electroelution Method

DNA from liver tissue was electrophoresed in a 0.7% agarose gel in 1/3 × TBE buffer (1 × TBE buffer is consisted of 89 mM Tris-borate and 2 mM EDTA) with a molecular weight standard marker, λ-HindIII digests. The gel block containing longer than 9 kilobase (kb) fragments of sample DNA (asterisked part in Fig. 1) was cut out and sealed

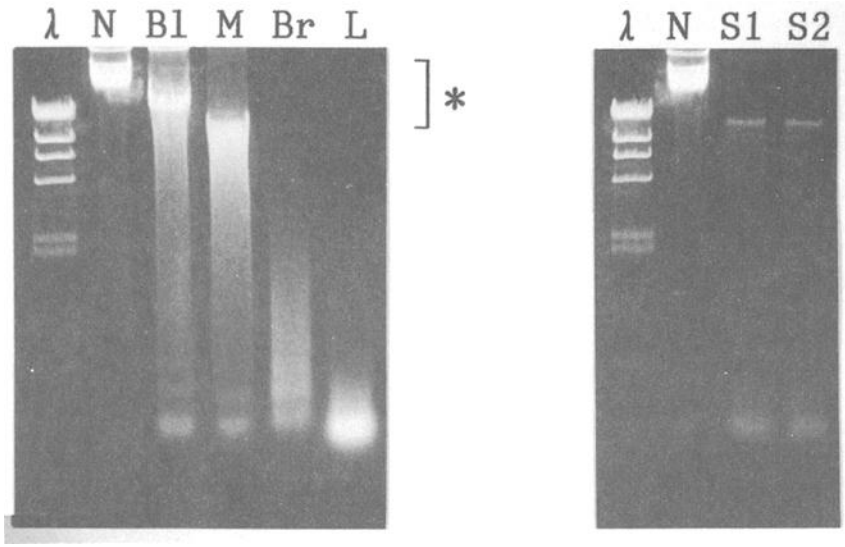

FIG. 1—*Electrophoresis in a 0.7% gel of DNA isolated from fresh peripheral leukocyte (N), from ventricular blood (Bl), muscle (M), brain (Br), and liver (L) from the dead woman, and from bloodstain (S1) and adipose tissue (S2) found in the accident scene. *Agarose gel block from which HMW DNA was recovered by electroelution. λ: λ-HindIII digests.*

in a dialysis tube. DNA was then electroeluted out of the gel [25], recovered using the A.S.A.P. column chromatography, and subsequently concentrated by ethanol precipitation or by ultrafiltration and ethanol precipitation.

Other Methods to Overcome Inhibition of PCR by Contaminants in the Bloodstain Sample

As one method to ensure PCR analysis of less-degraded and contaminated sample, DNA extracted from the experimentally prepared bloodstain by phenol/chloroform method was treated with Chelex® 100 (Bio-Rad, Richmond, CA) and Centricon 100 according to the method of Jung et al. [23]. Briefly, 200 μL 5% Chelex 100 containing 500 ng template DNA was incubated at 56°C for more than 30 min. The sample was vortexed for 10 s and boiled for 8 min. Following the microcentrifugation at 15 000 × g for 3 min, the supernatant was applied to Centricon 100, as described above. The recovered sample was used for PCR. As another method, 160 μg/mL bovine serum albumin (BSA) was added into the reaction mixture for PCR [19,21] after pretreatment of the sample with Centricon 100. The efficiencies of these procedures were compared to that of A.S.A.P. column chromatography and Centricon 100.

PCR Analysis

PCR was performed using 500 ng/reaction of DNA samples, if sufficient amounts of DNA were available. The nucleotide sequences of outer primers AMXY-1F and AMXY-2R for single PCR and nested primers AMXY-3FX, AMXY-3FY and AMXY-4R for dual PCR amplification of the amelogenin gene were shown elsewhere [8,15]. PCR was performed using AmpliTaq® DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in 50 μL PCR buffer (consisted of 10 mM Tris HCl, pH 8.3, 50 mM potassium chloride, 2.0 mM magnesium chloride) containing 200 μM each of deoxyribonucleoside-5′-triphosphates (dNTP) and 0.5 μM each of primers. From pure male genomic DNA, 788 bp Y and 977 bp X fragments were amplified by single PCR [8]. Then, 2 μL of the PCR product was used for the dual PCR and 674 bp Y′ and 863 bp X′ fragments were amplified [12]. Amplification using pure male and female DNA were performed as positive controls for appropriate PCR condition. X or X′ fragment of amelogenin gene also acted as internal controls of PCR [15]. Each PCR product was electrophoresed in an ethidium bromide-stained 1% agarose gel.

Results

DNA samples isolated from putrefied liver, bloodstain, and adipose tissue were slightly yellowish. Repeated phenol/chloroform treatment and ethanol precipitation did not clear them. The estimates for purities (OD_{260}/OD_{280}) of samples from liver, brain, muscle, blood, bloodstain and adipose tissue were 1.75, 1.64, 1.60, 1.88, 1.92, and 1.92, respectively, where the ratio of pure DNA is ≥1.8 [25]. The colored contaminants did not affect the ratio significantly. Spectrophotometric analysis of the liver sample showed the weak "shoulder" of spectrum at 390 to 400 nm (Fig. 2). The sample could be decolorized by Centricon treatment. Pyridine hemochromogen analysis of the dialysate of Centricon 100 obviously demonstrated the absorbance peak at 556 nm (Fig. 2), which was specific to pyridine hemochromogen derived from heme *b* [26]. The fourth derivative spectrophotometry, which enhances the absorbance change [27–29], validated the spectral peak at 556 nm (Fig. 2). The content of heme *b* in the liver DNA sample was 37.9 μM. The spectral features of pyridine hemochromogen derived from heme *a* from cytochrome

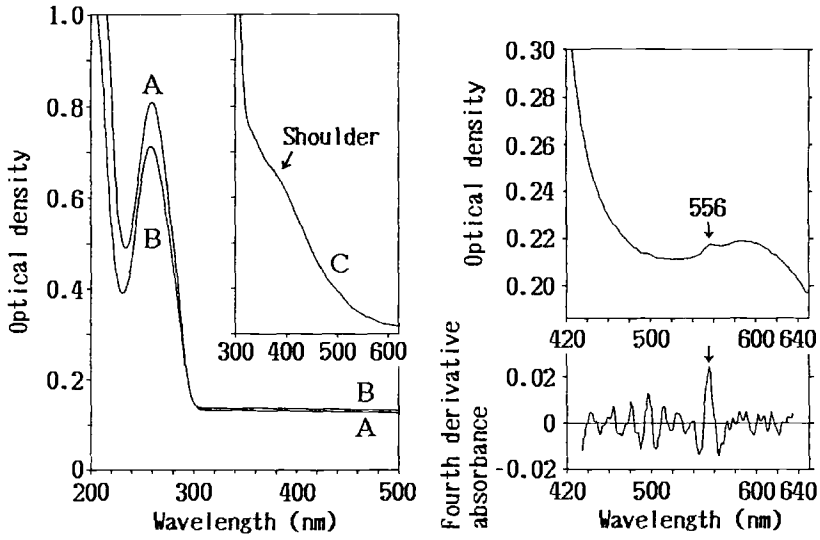

FIG. 2—*Left: Spectra of pure and impure DNA samples. A: 30 μg/mL pure DNA from fresh leukocytes, B and C: 30 and 3000 μg/mL of the same DNA sample from the liver tissue. Right: Standard (top) and the fourth derivative spectra (bottom) of pyridine hemochromogen derivatized from the impurity in the liver sample. The arrow indicates the absorbance peak specific to pyridine hemochromogen derived from heme* b.

oxidase (absorbance peak at 587 nm [*26*]) could not be identified. No impurities other than heme *b* were identified in this study.

When the samples from the tissues and stains were electrophoresed in a 0.7% agarose gel, a smeary band composed of degraded LMW DNA was observed in each lane (Fig. 1). The samples from ventricular blood and muscle tissue showed less degradation. The amelogenin gene could be amplified by PCR from these untreated samples, and no purification procedure described in this paper was performed. DNA from the liver tissue was most decomposed. When this sample was electrophoresed, HMW genomic DNA in the gel was faintly observed with the naked eye, whereas it was almost invisible in the photograph (Fig. 1). PCR did not succeed with the sample. Inhibition of PCR by some impurities was reported to be overcome by adding BSA [*19,21*], or by preparation using Chelex 100 and Centricon 100 treatments [*22,23*]. It was also reported that the use of formamide ensured the amplification of GC-rich sequence [*30*]. However, low-sensitivity amelogenin gene could not be detected from the highly degraded sample with these modifications. It was thus needed to remove LMW DNA, and the most degraded DNA from the liver tissue was then used to investigate the efficiencies of purification procedures.

By A.S.A.P. column chromatography and/or Centricon ultrafiltration, LMW DNA was not eliminated from the liver sample (Fig. 3). Among these treatments, Centricon 100 removed LMW DNA most effectively. From these samples, amelogenin gene could not be amplified at all even by dual PCR technique. Using the electroelution method, about 220 ng of HMW DNA was recovered from 300 μg of degraded liver DNA. This electroeluted sample was firstly purified by A.S.A.P. column chromatography instead of DEAE Sephacel gel filtration, since HMW DNA is not recovered by the latter method [*25*]. In addition, DEAE-cellulose membrane technique [*25*] was also not satisfactory for the recovery of HMW DNA from the gel. The electroeluted sample was divided into thirds and was concentrated either by ethanol precipitation, or by the Centricon device

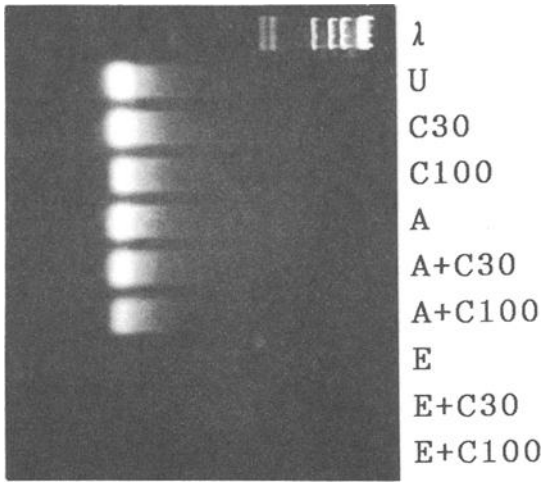

FIG. 3—*Electrophoresis in a 0.7% gel of DNA isolated from fresh leukocytes (N) and from putrefied liver before and after the purification procedures. U: untreated sample. C30, C100, A and E: samples purified by the ultrafiltration using Centricon 30 and 100, the A.S.A.P. column chromatography, and electroelution method, respectively.*

followed by ethanol precipitation. From half (about 35 ng) of each sample, the amelogenin gene (X′ fragment) could be amplified by dual PCR (Fig. 4), which was identified by comparing fragment lengths with the PCR product from pure male and female DNA. Little product was detected from the first reaction, since the starting concentration of template DNA was smaller than 250 ng/reaction [*8*]. Centricon treatment appeared to enhance the amplification of X′ fragment (Fig. 4). Moreover, Centricon 100 seemed to improve intensity of the PCR product more than Centricon 30.

DNA from the bloodstain and adipose tissue were less degraded than the untreated hepatic sample (Fig. 1). However, amelogenin gene could not be detected from 500 ng/

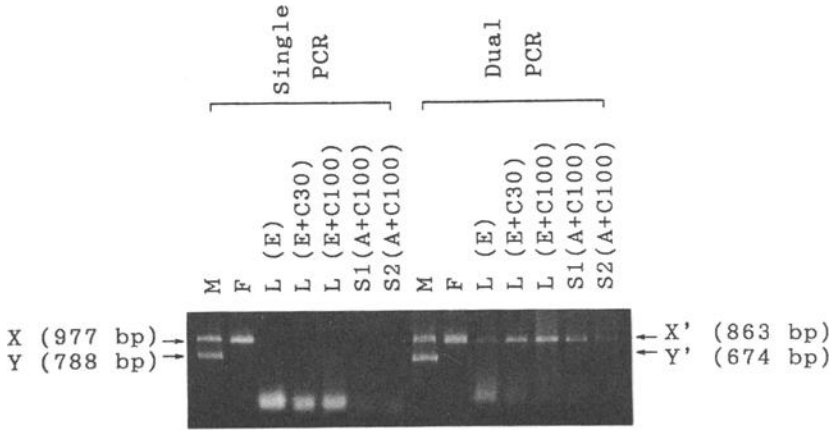

FIG. 4—*Electrophoresis in a 1% gel of amelogenin gene amplified using DNA from putrefied liver (L) from the dead woman, and bloodstain (S1) and adipose tissue (S2) found in the accident scene. C30, C100, A and E: see the legend to Fig. 2. M and F: PCR products from pure male and female genomic DNA, respectively.*

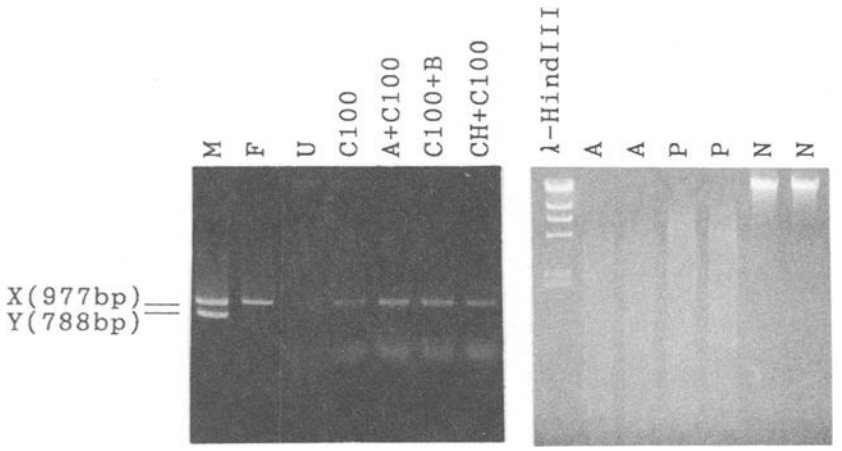

FIG. 5—*Left: Amplification of amelogenin gene from experimental bloodstain DNA. U: untreated. C100, A + C100, C100 + B and CH + C100: the sample treated by Centricon 100 alone, by A.S.A.P. column chromatography and Centricon 100, by Centricon 100 and BSA, or by Chelex 100 and Centricon 100, respectively. M and F: PCR products from pure male and female genomic DNA. Right: Electrophoresis in a 0.7% gel of DNA from an experimental bloodstain. The sample was incubated in the lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5% N-lauroylsarcosine, 50 mM EDTA, 50 μg/mL proteinase K) at 37°C overnight. The lysate was divided into four, and DNA was isolated by the column chromatography (A) or phenol/chloroform treatment (P). N: DNA extracted from fresh peripheral leukocyte by phenol/chloroform treatment.*

reaction of the untreated samples even by dual PCR (Fig. 4), probably due to the contamination. These samples were purified by the column chromatography and Centricon 100 treatment. The electroelution procedure was omitted because sufficient amount of HMW DNA was obtained. From these purified samples, the X′ fragment could be detected by dual PCR technique (Fig. 4).

For comparing several methods to overcome PCR inhibition by contaminants, DNA isolated by phenol/chloroform treatment from the experimentally prepared bloodstain was used; DNA was less degraded but yellowish-colored. By single PCR amplification of amelogenin gene, the X fragment was weakly amplified from the untreated sample (Fig. 5). Centricon 100 alone slightly improved the amplification. PCR product was more clearly detected after the pretreatments of the A.S.A.P. column chromatography, BSA addition [*19,21*], or Chelex 100, and Centricon 100 [*22,23*].

The DNA directly extracted from the experimental bloodstain by the A.S.A.P. kit was smaller in quantity [2.40 ± 0.120 μg (mean ± standard deviation for four determinations)] than that by phenol/chloroform treatment (4.01 ± 0.0679 μg) (Fig. 5). The extract by the former was more transparent, but the ratios of OD_{260}/OD_{280} were not different; 1.67 ± 0.0504 by the former while 1.69 ± 0.00497 by the latter.

Discussion

Contaminants from tissues such as hemoglobin and porphyrin are known to suppress the PCR amplification [*31*]. The other environmental contaminants are also likely to affect the reaction [*1–5*]. In addition, excess amounts of template DNA or PCR products can interfere with the reaction; misannealing between their complementary sequences will cause PCR products with various sizes. When tandemly repetitive genes are amplified, misannealing between the repeating units may occur more frequently and significantly [*32*]. Degraded LMW DNA was also reported to cause the misrepair [*33*]. Damaged

molecules would not be replicated at all and many lesions in them would slow down or arrest the enzyme reaction [*33*,*34*]. Incomplete extension products from damaged and misrepaired DNA would serve as primers, resulting in chimera of multiple templates [*34*]. Large amounts of decomposed LMW DNA might also act as physical barrier against the primer annealing. It was thus required to remove not only the impurities but also degraded LMW DNA from highly degraded samples. In this study, purification of the most degraded DNA extracted from putrefied liver tissue was explored for the PCR analysis of a low-sensitivity gene. As a model of the low-sensitivity target, the amelogenin gene for sex determination was used. Although we of course tried to avoid the contamination during the procedures [*35*,*36*], only female samples were analyzed in order to check the accidental adulteration of exogenous material, for example, male specimens from operators [*36*]. If the strategy for amplifying degraded templates for sex determination would be established using female samples, male degraded specimens would be detectable as well under the same analytical condition.

Yellowish DNA samples from putrefied liver, bloodstain, and adipose tissue indicated that the remains of cell materials were copurified with the DNA. Since the samples were decolorized by Centricon 30 or 100 that retains proteins with a molecular weight of more than 30 000 or 100 000, respectively, molecular weight of the impurity was likely to be smaller than 30 000. The spectral "shoulder" at 390 to 400 nm of the samples suggested the presence of heme compounds (Fig. 2), but was too weak to identify what the contaminant was. Then, pyridine hemochromogen analysis was performed for further investigation, showing the specific absorption of the heme *b* derivative. Heme *b* proteins in human tissues include hemoglobin, myoglobin, cytochrome *b*, cytochrome P-450, and catalase. It was not known which protein was the contaminant, but, at least, some of the heme *b* component was likely to be derived from hemoglobin. In DNA isolation procedures from the tissue, Proteinase K lysed the heme proteins. Then, heme *b* freed from the protein molecule (for example, globin protein in hemoglobin) might be derivatized to the water-soluble hematin form, that was copurified with DNA by phenol/chloroform treatment [*22*]. The molecular weight of the hematin is 633.49 and it can be dialyzed by Centricon 30 or 100. Chelex 100 preparation is also effective for the removal, possibly by binding iron ion to the chelating resin matrix [*22*,*23*]. Similarly, bloodstain extracts sometimes contain the porphyrin compound derived from hemoglobin [*22*].

DNA from ventricular blood and muscle tissue was less degraded (Fig. 1) as reported [*37*]. DNA from the liver tissue was most decomposed. Spleen and kidney were also reported to show rapid degradation of DNA [*37*]. The organs in the abdominal cavity seemed to putrefy rapidly due to the proliferation of enteric bacteria. The degradation of DNA was likely to progress with the putrefaction, while the mechanism was obscure. DNA samples from such putrefied tissues were likely to contain bacterial DNA [*3*–*5*].

Dual PCR amplification of the amelogenin gene did not succeed with the most degraded liver sample, even following that the interference by impurity was overcome by either adding BSA [*19*,*21*], Chelex 100 and Centricon 100 treatments [*22*,*23*], or the A.S.A.P. column chromatography. PCR only succeeded with the sample following the electroelution procedure. Subsequent Centricon treatment appeared to enhance the amplification of X′ fragment, and Centricon 100 seemed to improve the amplification more than Centricon 30 (Fig. 4). In PCR analysis, Centricon devices are frequently used for the removal of excess amounts of oligonucleotides and dNTP [*25*,*38*,*39*], and was applied to eliminate the remained LMW components after the electroelution step in this study. These findings suggested that the Centricon 100 was quite effective for the purpose, and that the removal of LMW components was very important to succeed the PCR analysis using such degraded sample DNA.

When DNA from experimentally prepared bloodstain was analyzed, PCR product was clearly detected after the pretreatments of A.S.A.P. column chromatography, BSA ad-

dition, or Chelex 100 (Fig. 5). BSA may bind soluble contaminants and block the interference with Taq polymerase activity [*21*]. Chelex 100 may bind the polyvalent metal ions of inhibitors in the samples [*22,23*]. Chelex 100 was also postulated to prevent the degradation during denaturation of DNA [*22*]. Since phenol/chloroform method sometimes copurified the contaminant, direct extraction of DNA from forensic specimens by the A.S.A.P. column chromatography or Chelex 100 would be more desirable. The Chelex procedure is more rapid, convenient and economical [*23*], during which DNA is denatured so as not to be suitable for restriction fragment length polymorphism (RFLP) analysis. On the other hand, greater amount of sample (up to 30 mL whole blood) can be applied to the A.S.A.P. column chromatography, and isolated DNA is also available for RFLP analysis. However, the quantity of DNA extracted by this kit was about 60% of that by phenol/chloroform treatment, and the A.S.A.P. kit was slightly expensive for routine work (¥40,400 for 10 purifications in Japan). For PCR analysis of small amounts of samples such as bloodstains, Chelex 100 was thus more suitable for isolating DNA. When highly degraded DNA was purified by the electroelution method, HMW DNA was recovered in greater volume (≥1 mL) of electrophoresis buffer with the impurity from agarose gel that would inhibit the enzyme reaction [*25*]. This impurity would not be eliminated by Chelex treatment, so that the column chromatography was required for the purification of electroeluted sample. Equilibrium centrifugation in cesium chloride-ethidium bromide gradients is also available for isolating and purifying sample DNA [*25,40*]. By this technique, the components with different densities are eliminated from DNA. However, this method was not investigated in this study, because it is time-consuming and will not remove LMW DNA with the same density.

This study demonstrated the possibility that degraded and contaminated DNA from forensic specimens were suitable for PCR analysis following the purification as described above, if trace amounts of HMW DNA remained. The electroelution method was, however, not only time-consuming but risky, providing a great chance for the sample contamination. Not only is always great care required [*35,36*], but rapid and riskless techniques should be developed and applied.

References

[*1*] McNally, L., Shaler, R. C., Baird, M., Balazs, I., De Forest, P., and Kobilinsky, L., "Evaluation of Deoxyribonucleic Acid (DNA) Isolated form Human Bloodstains Exposed to Ultraviolet Light, Heat, Humidity, and Soil Contamination." *Journal of Forensic Sciences*, Vol. 34, No. 5, Sept. 1989, pp. 1059–1069.

[*2*] McNally, L., Shaler, R. C., Baird, M., Balazs, I., Kobilinsky, L., and De Forest, P., "The Effects of Environment and Substrata on Deoxyribonucleic Acid (DNA): The Use of Casework Samples from New York City," *Journal of Forensic Sciences*, Vol. 34, No. 5, Sept. 1989, pp. 1070–1077.

[*3*] Prinz, M. and Berghaus, G., "The Effect of Various Stain Carriers on the Quality and Quantity of DNA Extracted from Dried Bloodstains," *Zeitschrit für Rechtsmedizin*, Vol. 103, 1990, pp. 191–197.

[*4*] Adams, D. E., Presley, L. A., Baumstark, A. L., Hensley, K. W., Hill, A. L., Anoe, K. S., Campbell, P. A., McLaughlin, C. M., Budowle, B., Giusti, A. M., Smerick, J. B., and Baechtel, F. S., "Deoxyribonucleic Acid (DNA) Analysis by Restriction Length Polymorphisms of Blood and Other Body Fluid Stains Subjected to Contamination and Environmental Insults," *Journal of Forensic Sciences*, Vol. 36, No. 5, Sept. 1991, pp. 1284–1298.

[*5*] Comey, C. T. and Budowle, B., "Validation Studies on the Analysis of the HLA DQα Locus Using the Polymerase Chain Reaction," *Journal of Forensic Sciences*, Vol. 36, No. 6, Nov. 1991, pp. 1633–1648.

[*6*] Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, C. T., Mullis, K. B., and Erlich, H. A., "Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," *Science*, Vol. 239, Jan. 1986, pp. 7569–7580.

[7] Kogan, S. C., Doherty, M., and Gitschier, J., "An Improved Method for Prenatal Diagnosis of Genetic Diseases by Analysis of Amplified DNA Sequences," *New England Journal of Medicine*, Vol. 317, No. 16, Oct. 1987, pp. 985–990.

[8] Akane, A., Shiono, H., Matsubara, K., Nakahori, Y., Seki, S., Nagafuchi, S., Yamada, M., and Nakagome, Y., "Sex Identification of Forensic Specimens by Polymerase Chain Reaction (PCR): Two Alternative Methods," *Forensic Science International*, Vol. 49, No. 1, Jan./Feb. 1991, pp. 81–88.

[9] Witt, M. and Erikson, R. P., "A Rapid Method for Detection of Y-Chromosomal DNA from Dried Blood Specimens by the Polymerase Chain Reaction," *Human Genetics*, Vol. 82, No. 3, Jan. 1989, pp. 271–274.

[10] Gaensslen, R. E., Karen, M., Berka, M. S., Grosso, D. A., Ruano, G., Pagliaro, E. M., Messina, D., and Lee, H. C., "A Polymerase Chain Reaction (PCR) Method for Sex and Species Determination with Novel Controls for Deoxyribonucleic Acid (DNA) Template Length," *Journal of Forensic Sciences*, Vol. 37, No. 1, Jan. 1992, pp. 6–20.

[11] Yamamoto, Y., Nakanishi, A., Semba, S., and Ishizu, H., "Sex Determination of Bloodstains by Polymerase Chain Reaction (PCR) of Y-Chromosome-Specific Alphoid Repeat Sequences," *Acta Criminologie et Medicinae Legalis Japonica*, Vol. 57, No. 5, Oct. 1991, pp. 187–191.

[12] Akane, A., Seki, S., Shiono, H., Nakamura, H. Hasegawa, M., Kagawa, M., Matsubara, K., Nakahori, Y., Nagafuchi, S., and Nakagome, Y., "Sex Determination of Forensic Samples by Dual PCR Amplification of an X-Y Homologous Gene," *Forensic Science International*, Vol. 52, No. 2, Jan. 1992, pp. 143–148.

[13] Nakahori, Y. Hamano, K., Iwaya, M., and Nakagome, Y., "Sex Identification by Polymerase Chain Reaction Using X-Y Homologous Primer," *American Journal of Medical Genetics*, Vol. 39, 1991, pp. 472–473.

[14] Nakahori, Y., Takenaka, O., and Nakagome, Y., "A Human X-Y Homologous Region Encodes 'Amelogenin'," *Genomics*, Vol. 9, 1991, pp. 264–269.

[15] Nakagome, Y., Seki, S., Nagafuchi, S., Nakahori, Y., and Sato, K., "Absence of Fetal Cells in Maternal Circulation at a Level of 1 in 25,000," *American Journal of Medical Genetics*, Vol. 40, 1991, pp. 506–508.

[16] Mullis, K. B. and Faloona, F. A., "Specific Synthesis of DNA in vitro via a Polymerase-Catalyzed Chain Reaction," *Methods in Enzymology*, Vol. 155, 1987, pp. 335–350.

[17] Erlich, H. A., Gelfand, D., and Sninsky, J. J., "Recent Advances in the Polymerase Chaintion," *Science*, Vol. 252, June 1991, pp. 1643–1651.

[18] Higuchi, R., von Beroldingen, C. H., Sensabaugh, G. F., and Erlich, H. A., "DNA Typing from Single Hairs," *Nature*, Vol. 332, April 1988, pp. 543–546.

[19] Hagelberg, E., Sykes, B., and Hedges, R., "Ancient Bone DNA Amplified," *Nature*, Vol. 342, 1989, p. 485.

[20] Walsh, P. S., Fildes, N., Louis, A. S., and Higuchi, R., "Report of the Blind Trial of the Cetus AmpliTaq HLA DQα Forensic Deoxyribonucleic Acid (DNA) Amplification and Typing Kit," *Journal of Forensic Sciences*, Vol. 36, No. 5, Sept. 1991, pp. 1551–1556.

[21] Hochmeister, M. N., Budowle, B., Borer, U. V., Eggmann, U., Comey, C. T., and Dirnhofer, R., "Typing of Deoxyribonucleic Acid (DNA) Extracted from Compact Bone from Human Remains," *Journal of Forensic Sciences*, Vol. 36, No. 6, Nov. 1991, pp. 1649–1661.

[22] Walsh, P. S., Metzger, D. A., and Higuchi, R., "Chelex® 100 as a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material," *Biotechniques*, Vol. 10, 1991, pp. 506–513.

[23] Jung, G. M., Comey, C. T., Baer, D. B., and Budowle, B., "Extraction Strategy for Obtaining DNA from Bloodstains for PCR Amplification and Typing of the HLA-DQα Gene," *International Journal of Legal Medicine*, Vol. 104, No. 3, July 1991, pp. 145–148.

[24] Akane, A., Matsubara, K., Shiono, H., Yuasa, I., Yokota, S.-I., Yamada, M., and Nakagome, Y., "Paternity Testing: Blood Group Systems and DNA Analysis by Variable Number of Tandem Repeat Markers," *Journal of Forensic Sciences*, Vol. 35, No. 5, Sept. 1990, pp. 1217–1225.

[25] Sambrook, J., Fritsch, E. F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, New York, 1989.

[26] Rieske, J. S., "The Quantitative Determination of Mitochondria Hemoproteins," *Methods in Enzymology*, Vol. 10, R. W. Estabrook and M. E. Pullman, Eds., Academic Press, New York, 1967, pp. 488–493.

[27] Fukui, Y., Matsubara, M., Takahashi, S., and Matsubara, K., "A Study of Derivative Spectrophotometry for the Determination of Carboxyhemoglobin in Blood," *Journal of Analytical Toxicology*, Vol. 8, Nov./Dec. 1984, pp. 277–281.

[28] Fukui, Y., Matsubara, M., Akane, A., Hama, K., Matsubara, K., and Takahashi, S., "Determination of Carboxyhemoglobin in Heated Blood—Sources of Error and Utility of Deriv-

ative Spectrophotometry," *Journal of Analytical Toxicology*, Vol. 9, March/April 1985, pp. 81–84.

[29] Fukui, Y., Akane, A., Takahashi, S., Matsubara, K., and Maseda, C., "Derivative Spectrophotometric Studies on Cytotoxicity Effects of Carbon Monoxide," *Forensic Science International*, Vol. 33, 1987, pp. 75–82.

[30] Comey, C. T., Jung, J. M., and Budowle, B., "Use of Formamide to Improve Amplification of HLA DQα Sequences," *Biotechniques*, Vol. 10, 1991, pp. 60–61.

[31] Higuchi, R., "Simple and Rapid Preparation of Samples for PCR," *PCR Technology: Principles and Applications for DNA Amplification*, H. E. Erlich, Ed., Stockton Press, New York, 1989, pp. 31–38.

[32] Jeffreys, A. J., Wilson, V., Neumann, R., and Keyte, J., "Amplification of Human Minisatellites by the Polymerase Chain Reaction: Towards DNA Fingerprinting of Single Cells," *Nucleic Acids Research*, Vol. 16, No. 23. Dec. 1988, pp. 10 953–10 971.

[33] Pääbo, S., Higuchi, R. G., and Wilson, A. C., "Ancient of DNA and the Polymerase Chain Reaction," *Journal of Biological Chemistry*, Vol. 264, No. 17, June 1989, pp. 9709–9712.

[34] Rogan, P. K. and Salvo, J., "Study of Nucleic Acids Isolated from Ancient Remains," *Yearbook of Physical Anthropology*, Vol. 33, 1990, pp. 195–214.

[35] Kwok, S. and Higuchi, R., "Avoiding False Positives with PCR," *Nature*, Vol. 339, May 1989, pp. 237–238.

[36] Kitchin, P. A., Szotyori, Z., Fromholc, C., and Almond, N., "Avoidance of False Positives," *Nature*, Vol. 344, 1990, p. 201.

[37] Bär, W., Kratzer, A., Mächler, M., and Schmid, W., "Postmortem Stability of DNA," *Forensic Science International*, Vol. 39, 1988, pp. 59–70.

[38] Higuchi, R., Krummel, B., and Saiki, R. K., "A General Method of in vitro Preparation and Specific Mutagenesis of DNA Fragments: Study of Protein and DNA Interactions," *Nucleic Acids Research*, Vol. 16, No. 15, Aug. 1988, pp. 7351–7367.

[39] Mihovilovic, M. and Lee, J. E., "An Efficient Method for Sequencing PCR Amplified DNA," *Biotechniques*, Vol. 7, No. 1, 1989, pp. 14–16.

[40] Weeks, D. P., Beerman, N., and Griffith, O. M., "A Small-Scale Five-Hour Procedure for Isolating Multiple Samples of CsCl-Purified DNA: Application to Isolations from Mammalian, Insect, Higher Plant, Algal, Yeast, and Bacterial Sources." *Analytical Biochemistry*, Vol. 152, 1986, pp. 376–385.

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