TECHNICAL NOTE

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Detection of D1S80 (pMCT118) Locus Polymorphism Using Semi-nested Polymerase Chain Reaction in Skeletal Remains

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ABSTRACT: We evaluated the usefulness of a semi-nested polymerase chain reaction (PCR) method for detecting D1S80 (pMCT118) locus polymorphisms of DNA extracted from old skeletal remains. The semi-nested PCR has been applied to the amplification of D1S80 nucleic acid sequences. For amplification of the locus D1S80, a pair of oligonucleotide primers have been used widely as described by Kasai et al. We have designed another set of primers for semi-nested PCR. This method resulted in D1S80-VNTR detection from low-titered DNA isolated from old skeletal remains. The first and second step PCR achieved amplification from as little as 10 ng and 10 pg of template DNA, respectively. Specificity and sensitivity of the amplification products was markedly improved by semi-nested PCR. In DNA extracted from biological samples, this method took about 5 hours to amplify the target DNA and 3 hours for electrophoretic separation. We demonstrated that this semi-nested PCR method was superior in sensitivity to conventional 1-step standard amplification for VNTR typing of the D1S80 locus.

KEYWORDS: forensic science, skeletal remains, semi-nested PCR, D1S80 (pMCT118), VNTR polymorphism, forensic identification, human identification

Amplification of the VNTR locus from DNA in skeletal remains is sometimes difficult due to postmortem alterations such as base modification and degradation. Except in extraordinarily well-preserved samples, the amount of extractable DNA is generally very little and detection of a locus even using the polymerase chain reaction (PCR) is restricted. In the forensic field, PCR amplification of VNTR loci such as D1S80 (pMCT118) [*1,2*], Apo B [*3*], D17S5 (YNZ 22) [*4*], COL2A1 [*5,6*] has been shown to be applicable for personal identification and paternity testing. Particularly, the D1S80 locus has a relatively large number of polymorphisms, and exhibits high discrimination power (DP) [*7*]. In this study, we evaluated the usefulness of a semi-nested PCR method for detecting D1S80 (pMCT118) locus polymorphisms in DNA extracted from the trace amounts in old skeletal remains.

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Materials and Methods

Sample Collection

Blood Samples—Fresh blood samples from healthy donors were collected under sterile conditions and stored at 4°C until DNA extraction.

Hard Tissues—We collected several skull fragments approximately 10 mm in diameter each and a single tooth (pre-molar) from corpses scheduled for autopsy.

DNA Extraction

DNA from fresh blood samples was isolated by the phenol/chloroform procedure [*8*].

DNA was extracted from hard tissues as described previously [*9,10*]. Briefly, we cleaned bone and tooth surfaces with a brush and washed them in a supersonic processor to remove any foreign particles. Bone marrow was separated from compact bone by placing the specimen in detergent and then shaking vigorously in an SR shaker system-SR300 (Shimadzu). Compact bone and tooth with the pulp were placed into liquid nitrogen [*11*] for one minute, and then pulverized with a hammer in a sterilized polyethylene bag. Bone marrow was then digested with proteinase K (200 μg/mL) in lysis buffer (3 mL including 50 mM Tris-HCl, 100 mM NaCl, 0.5% SDS, 1 mM EDTA) at 50°C for 4 h. The bone and tooth powder were dissolved in the lysis buffer containing a high concentration (0.23 M) of EDTA:2Na/4Na (pH 7.4) for decalcification at room temperature for 48 h. After dialysis against 10 mM Tris-HCl (pH 8.0) and 5 mM EDTA, the bone and tooth extracts were digested with proteinase K (100 μg/mL) in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5% SDS, 1 mM EDTA) at 50°C for 4 h. DNA was extracted by the phenol/chloroform procedure [*8*], precipitated with ethanol, and then dried and stored at −20°C in 10 mM Tris-HCl (pH 7.6).

Extracted DNA was quantified fluorometrically at 260 nm (Beckman-DU640).

PCR

The three oligonucleotide primers P1a, P1b and P2 were synthesized for semi-nested PCR of MCT118. The sequences of the three primers were as follows:

P1a: 5′-CCATGAGGCGCTGAGAGAAAC-3′

P1b: 5′-GAAACTGGCCTCCAAACACTGCCCGCCG-3′

P2: 5′-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3′

The internal primer pair made up of P1b and P2 were described previously by Kasai et al. for standard 1-step PCR [*1*]. The external primer pair P1a and P2 were newly designed for semi-nested PCR based on the known sequence of D1S80. Primer P1a corresponded to a sequence 16 bp downstream from P1b, and P2 was used during the first and second amplification steps. In the first round of PCR, the external pair of primers (P1a and P2) was used: 1 pg to 100 ng of total DNA was subjected to 27 cycles of amplification in a 100 μL reaction mixture containing: 1 pg to 100 ng DNA (template), 2.5 units Taq-Polymerase (Promega), each primer at 0.5 μM, 200 μM d-NTPs, 50 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 250 ng/mL bovine serum albumin (BSA).

Initial denaturation was at 94°C for 2 min, followed by 27 cycles of 94°C denaturation (1 min), 65°C annealing (1 min), 72°C extension (2 min), with a final 72°C extension of 5 min.

To remove excess primers and dNTPs, the amplification mixture was centrifuged in a Microcon-100 microconcentrator (Amicon). The amplification product was pelleted by spinning in a microcentrifuge for 30 min at 5000 g, and the resulting pellet was then washed in 75% ethanol twice and vacuum dried briefly. The pellet was then resuspended in 100 μL of dH_2O.

The amplified DNA was subjected to a second round of amplification, using the semi-nested primer pair (P1b and P2). In this procedure, one of the primers (P2) from the first PCR was also used. The second round of PCR was performed under the following conditions: one μL of the first amplification product was used as the template in 50 μL reaction mixture. Constituents and concentrations of reaction buffer and thermocycles were the same as in the first-round PCR, except that the number of cycles was reduced to 20.

Ten-μL of amplified product was electrophoresed in a 1.5% of agarose gel (FMC Bio Products) for 3 hours at 150V, and directly visualized with ethidium bromide under UV illumination. Each band size was calculated with the logarithmic regression curve determined by mobility of the standard MW marker (100 base-pair ladder; Pharmacia-LKB).

Results

We first confirmed formation of bands by the first PCR using DNA from the fresh blood sample. In the first PCR using outer primers P1a and P2, amplification fragments were 16 bp longer than those in the second PCR in which the internal primer pair was used (Fig. 1). This result was consistent with previously described sequence data, demonstrating that the first amplification step had been successful in generating specific bands.

The lower limit of template concentration in the first PCR to generate specific bands was almost as low as 10 ng (Figs. 1, 2),

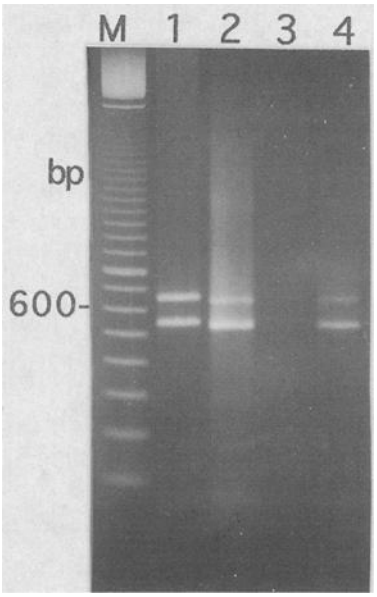

FIG. 1—*Amplification by first- and second-round PCR. The first round of PCR was carried out using standard methods (primers P1a; P2), and the product generated was used as template in the second round of PCR (primers P1b; P2). Amplified products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. M: 100 base-pair ladder (Pharmacia-LKB); lanes 1 and 2: first- and second-round amplification products from 100 ng template DNA obtained from fresh blood sample (allele 24/32). Lanes 3 and 4: first- and second-round amplification products from 1 ng of template DNA obtained from fresh blood sample (allele 24/32).*

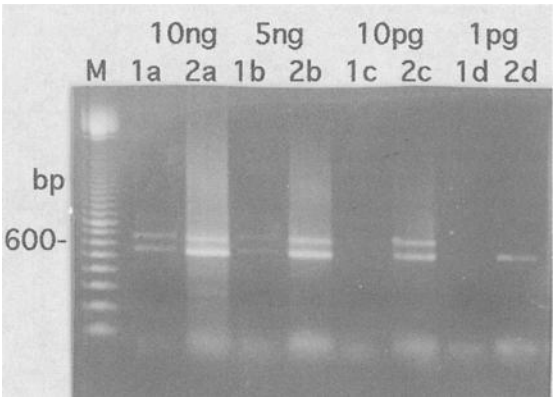

FIG. 2—*Checking the sensitivity of first-and second-rounds of PCR. M: 100 base-pair ladder (Pharmacia-LKB), a lanes: 10 ng (using 10 ng of template DNA), 1a: first-round PCR with primers P1a and P2, 2a: second-round PCR with primers P1b and P2, b lanes: 5 ng (using 5 ng of template DNA), 1b: first-round PCR with primers P1a and P2, 2b: the second-round PCR with primers P1b and P2, c lanes: 10 pg (using 10 pg of template DNA), 1c: first PCR with primers P1a and P2, 2c: second-round PCR with primers P1b and P2, d lanes: 1 pg (using 1 pg of template DNA), 1d: first PCR with primers P1a and P2, 2d: second PCR with primers P1b and P2. The allele of the sample was 24/30 repeats.*

while semi-nested PCR successively amplified by the inner primer pair could generate specific bands from as concentrations as low as 10 pg of template DNA. However, one of the allele pair was sometimes preferentially amplified when less than 1 pg of template was used (Fig. 2; lane: 1 pg-2d). Semi-nested PCR was 1000 times more sensitive than standard PCR (Fig. 2), and greater specificity and sensitivity could be achieved using semi-nested primer pairs.

For clear amplification in the second-round PCR, a 1 μL aliquot of the first reaction mixture was sufficient. Microconcentration of the first reaction mixture proved to be an effective means of preventing non specific amplification [*12*].

Under the optimum conditions outlined above, semi-nested PCR was performed to amplify the D1S80 locus using old skeletal extracts that could not be amplified by standard 1-step PCR. Six of seven cases could be amplified clearly (Fig. 3).

Discussion

In criminal investigation, examination of severely degraded and contaminated DNA extracted from biological evidence left for a long period is inevitable.

In addition, identification of corpses in a severe state of decay is frequently difficult but particularly important in homicide enquiries. Therefore, detection of old DNA is essential to the investigation and resolution of such crimes. Greater sensitivity cannot reasonably be expected without development of a method more sensitive than the standard PCR detection such as nested PCR [*13*] or hybridization with a labeled probe [*14*].

For identification from old degraded DNA, microsatellite regions are of special interest and meet the requirements for the present discussion [*15,16*]. PCR amplification of microsatellite regions is relatively easy to perform even from old DNA. However, size determination is sometimes contradicted because amplified fragments would not migrate according to their moleculor weights in the polyacrylamide gel electrophoresis as we had reported previously [*17*]. On the other hand, so-called AMPFLP (amplifiable fragment length polymorphism) detection is easy to perform and allows DNA typing by agarose electrophoretic separation to distinguish a particular kind of tandem repeat with a basic unit of roughly more than 15 bp [*18*]. Therefore, we had developed a nested-PCR technique to amplify COL2A1-VNTR region in skeletal remains and reported the extreme sensitivity of this method [*12*].

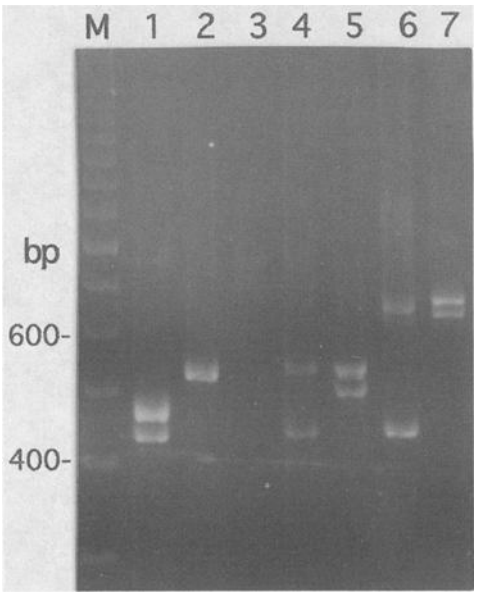

FIG. 3—*Amplification and detection of D1S80 locus polymorphisms from old skeletal remains by semi-nested PCR. M: 100 base-pair ladder (Pharmacia-LKB), lane 1: Bone marrow DNA extracted from 4-month-old skeleton (allele 18/20), lane 2: bone marrow DNA extracted from 8-month-old skeleton (allele 24/24), lane 3: bone DNA extracted from 1-year-old skeleton (no amplification), lane 4: Bone DNA extracted from 3-year-old skeleton (allele 18/24), lane 5: bone DNA extracted from 6-year-old skeleton (allele 22/24), lane 6: tooth DNA extracted from 4-year-old skeleton (allele 18/31), lane 7: tooth DNA extracted from 10-year-old skeleton (allele 30/32).*

In this study, we applied semi-nested PCR to amplify the D1S80 locus. This method has several advantages for forensic DNA analysis. First, this assay is easy to perform and takes less than 8 hours to complete two rounds of PCR. When extractable DNA was gained from well-preserved samples, VNTR typing could be established using only the first-step PCR.

Second, electrophoretic separation in an agarose gel can be easily and rapidly performed because alleles differ by 16-bp. Moreover, the number of polymorphisms found in the Japanese population are so abundant that it allows individual identification with great certainty [*18*].

Semi-nested PCR using two primer pairs exhibited extreme sensitivity, starting with as little as 10 pg of template DNA. Therefore, as long as 10 pg of intact sequence can be isolated as a template, the DNA-typing can be successfully performed by agarose gel electrophoresis and ethidium bromide staining. On the other hand, because this technique is extremely sensitive, excessive amplification or differential amplification sometimes occurred in contaminated samples. Thus, special care should be taken to maintain a strict foreign DNA-free environment.

In cases where total DNA applied to semi-nested PCR contained less than roughly 5 pg of template DNA (approximately one genome of a single cell), one of the alleles was preferentially amplified while the other was not amplified. In these cases, we could identify only one allele. Until reamplification is performed, therefore, to ascertain whether the sample DNA was heterozygous, we cannot conclusively state that it was homozygous.

The reason why semi-nested PCR is more sensitive has not been investigated throughly in our study. It is speculated that the high sensitivity of the method was due to diluted effect of an admixture of extracted DNA through two-step amplification. Furthermore, effective denaturation and annealing in the second round of PCR is induced by smaller uniformly sized templates obtained from the first amplification mixture. However, it remained as unsolved question why a total of 50 cycles (25 × 2) of PCR using one pair of primers twice was not effective in rising sensitivity of amplification (data not shown).

Semi-nested PCR of D1S80 from skeletal extracts represents a useful technique in addressing forensic identification of whole biological evidence in criminal investigation. Our findings demonstrated the utility of semi-nested PCR as a rapid, non-invasive method for the routine laboratory forensic identification.

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