New Method of PCR Amplification of Two Human Minisatellite Loci and Reliable Method of DNA Isolation

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 127, No 3, pp. 357-360, March, 1999
Original article submitted August 17, 1998

A method of DNA isolation and purification from highly contaminated sources usual for forensic medicine and criminalistic practice was developed. Oligonucleotide primers were synthesized and the conditions of PCR amplification were optimized for two human minisatellite (VNRT) loci — apolipoprotein B and D1S30 widely used in PCR-based DNA typing. The proposed procedure of DNA isolation includes standard treatment with proteinase K followed by phenol-chloroform extraction and further purification from cations and low molecular weight compounds on a Dowex/Sephadex G-50 minicolumn. The length of the primers and optimal PCR conditions determine stability and specificity of amplification of the VNTR-loci. The sensitivity of the proposed method is 2-4 ng DNA template in the reaction mixture.

Key words: *DNA isolation; DNA typing; human minisatellite loci; PCR; oligonucleotide primers*

Polymerase chain reaction (PCR) is now widely used not only in basic molecular biology or genetics but also in forensic medicine and criminalistics. The PCR technique is useful for DNA fingerprinting, where the quality of DNA isolation from different carriers is of critical importance. Both successful PCR procedure and unambiguous personal identification depends on the purity of DNA samples rather than on DNA content. Recently a number of methods of DNA isolation from different specimens have been developed [2,3,5, 7,8,10,11,14].

Treatment of the samples with proteinase K and sodium dodecyl sulfate (SDS) followed by phenol-chloroform extraction and DNA precipitation with ethanol is the most common method of DNA isolation in forensic medicine and criminalistics [3,8,10]. However, this method suits only for analysis of whole blood [3,8,10] or fresh blood and sperm spots on cotton materials [11,14], whereas experts often deal with highly contaminated and degraded DNA samples. Special kits for fine DNA purification are now available from different manufacturers [2]. When choosing the method for DNA isolation and purification, one must take into account the nature of a carrier as well as the duration and conditions of its storage. Several methods of DNA isolation from bones [5], blood spots on different types of fabrics [11], and small biological specimens [14] have been recently described. An original method of DNA isolation without extraction with organic solvents and proteinase K treatment has been proposed by D. K. Lahiri *et al.* [7].

We developed a simple and timesaving method of DNA isolation for subsequent PCR amplification. This method can be successfully used to samples most often seen in forensic medicine or criminalistics (jean cloth, putrefactive corpse material etc.). Particular emphasis has been placed on the specificity and stability of PCR amplification for two minisatellite (VNTR) loci, apolipoprotein B (apoB) and D1S30, which are often used in human DNA typing. For this purpose the lengths and sequence of the corresponding oligonucleotide primers as well as PCR protocols were optimized.

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0007-4888/99/0003-0324$22.00

MATERIALS AND METHODS

Deoxynucleoside triphosphates were synthesized at the Institute of Bioorganic Chemistry of the Russian Academy of Sciences (Pushchino). Taq DNA polymerase was prepared by authors. Other chemicals (salts, resins, etc.) were from Serva, Sigma and Boehringer Mannheim.

Human DNA samples were isolated from the peripheral blood and contaminated biological specimens (jean and synthetic cloths) according to [3,8,10] with some modifications. Blood spots were extracted as describes previously [2].

A two-steps method of DNA isolation and purification was used. First, blood samples (25 μl) or blood spots on the cloth (1×1 cm) were suspended in 400 μl of 10 mM Tris-HCl (pH 8), containing 100 mM NaCl, 10 mM EDTA, 2% SDS, and 0.2 mg proteinase K and incubated at 65°C for 3 h (all chemicals were from Sigma). To separate blood spot from the pieces of cloth the sample was placed into a 200-μl tip and centrifuged. The cloth was trapped inside a tip, while DNA was collected in a 1.5-ml centrifuge tube. All subsequent manipulations were as for the whole blood: 450 μl rectified phenol equilibrated with 0.1 mM Tris-HCl (pH 8) was added, each sample was agitated for 1 min, centrifuged for 5 min in a microcentrifuge at a maximum speed. The aqueous phase was collected and extraction with chloroform:isoamyl alcohol (24:1) was performed. The resulting aqueous phase was mixed with 0.1 volume of 3 M sodium acetate (pH 5.6) and 0.6 volume of isopropanol, tubes were vigorously agitated and incubated at room temperature for 10 min. DNA was precipitated by centrifugation in a microcentrifuge for 10 min at a maximum speed. The pallet was washed with ice-cold 75% ethanol, air-dried, and redissolved in 30-50 μl deionized water.

Further purification of the DNA preparations obtained from highly contaminated samples was carried out using Dowex and Sephadex G-50 minicolumns. The columns were made from 0.5 ml Eppendorf tubes with a 1-mm opening in the bottom covered with a GF/A filter (2×2 mm); 300-400 μl Sephadex G-50 and 50 μl Dowex suspensions were layered on the filter. Sephadex G-50 was preliminary equilibrated with 10 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA. Dowex was converted into a Na-form and thoroughly washed with 10 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA. The column was dried by centrifugation for 5 min at 4000 rpm. A 25-50-μl DNA sample was layered and centrifuged for 5 min at 4000 rpm (the column was placed into an 1.5-μl Eppendorf tube). The collected material was used for PCR amplification.

The concentration of human DNA in samples was measured as described elsewhere [2].

PCR amplification for apoB and D1S30 were performed as described previously [1,6]. Reaction mixture (25 μl) contained 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 0.02% Tween-20, 0.2 mM of each deoxynucleoside triphosphate, 10-100 ng DNA, 0.25 μM of each primer and 2.5 U Taq polymerase. The RCR was carried out on a MS-2 thermocycler (DNA-technology, Russia) using the following programming: for apoB — one 3-min cycle at 94°C, 30 cycles at 94°C for 40 sec, at 62°C for 50 sec, and at 72°C for 2 min, followed by a 5-min extension step at 72°C; for D1S30 — 3-min denaturation cycle at 94°C, followed by 30 cycles (94°C for 40 sec, 66°C for 40 sec, 72°C for 2 min) and a 5-min extension cycle at 72°C.

Electrophoretic separation of PCR products was carried out in 2% agarose gel in Tris-borate buffer [9]. The alleles of the two loci were labeled with specially synthesized markers (Fig. 1). The electrophoresis was performed at 8 V/cm; bromphenol blue migrated by 12-15 cm from the start.

Both standard [1] and originally designed primers for amplification of the apoB locus were used. The original primers were 5'-ATGGAAACGGAGAAAT TATGGAGG (sense) and 5'-CCTTCTCACTTGGCA AATACAATTCC (antisense). These primers were synthesized with an ASM-102U automatic synthesizer (Biosset, Novosibirsk, Russia) using a previously described solid-phase method. The primers for the D1S30 locus also had both the standard [6] and original sequences — forward 5'-AGTGAAGTGCACAGGAG GGCAAGGCGGTC and reverse 5'-GTGCCCACA GTCTTTATTCTTCAGCGTTC.

PCR amplification of all DNA samples was curried out in the presence or absence of bovine albumin (fraction V, Sigma) at final concentrations from 50 to 500 μg/ml.

RESULTS

The DNA samples obtained from biological specimens often contain organic and nonorganic admixtures which can inhibit DNA polymerize reaction [12,13]. In these cases extraction with organic solvent followed by Centricon-100 treatment is thought to be the best method for DNA purification [13]. We believe that this method minimizes DNA losses and yields the most stable results. On the other hand this relatively tedious technique requires special equipment and is of little value for DNA purification from highly contaminated samples. We propose a simple two-step method of DNA isolation and purification from highly contaminated biological specimens. The first step includes standard proteinase K treatment followed by phenol-chloroform

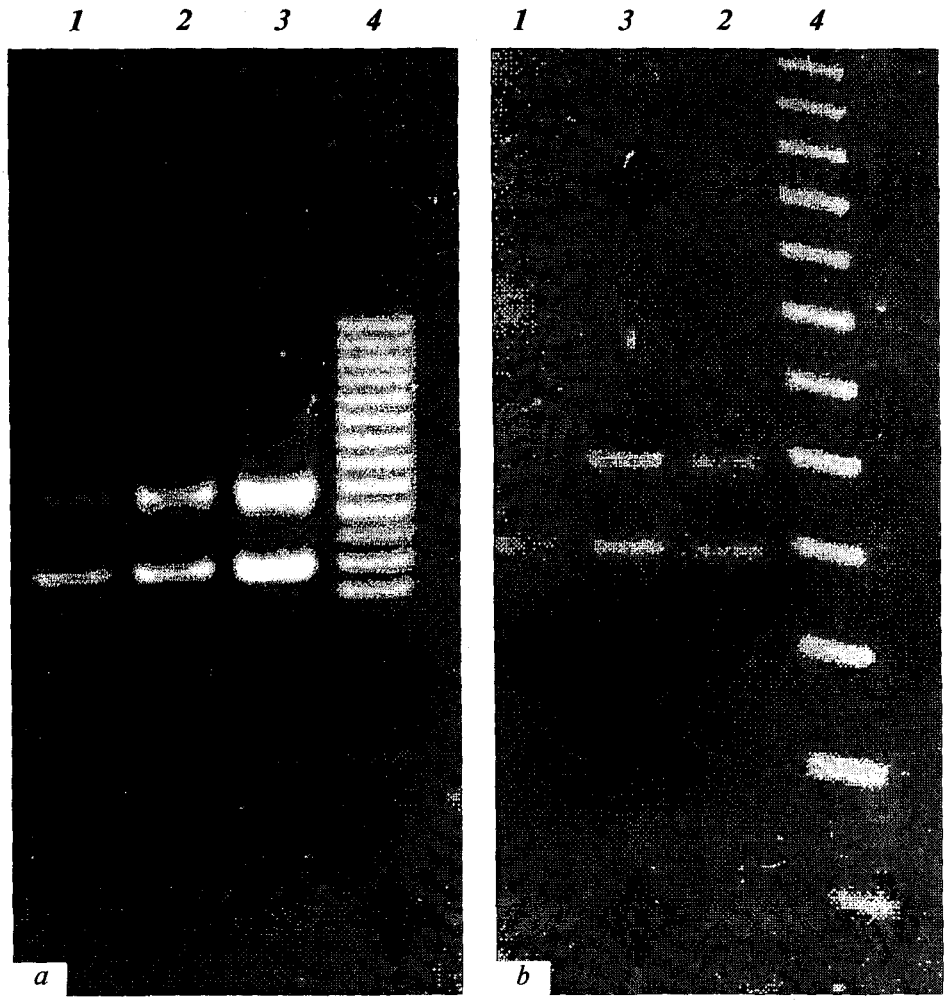

Fig. 1. Electrophoresis of PCR products for apolipoprotein B (*a*) and D1S30 (*b*) loci in 2% agarose gel. Standard (*1*) and lengthened (*2*) primers; lengthened primers together with albumin up to 160 μg/ml (*3*) and markers (*4*). Reaction mixture contained 2 ng DNA.

extraction [3,8,10]. Then the sample is purified on a Dowex/Sephadex G-50 minicolumn. The upper layer (Dowex) removes basic impurities (cations), while gelfiltration though Sephadex G-50 separates low molecular weight compounds. DNA samples were isolated from highly contaminated carriers containing different dyes (i.g., indigo) and prosthetic DNA polymerase inhibitors (i.g., hemoproteins) The latter are also present in some biological specimens, i.e. putrefactive corpse materials. PCR amplification of these DNA samples is a difficult practical problem. However, the stability of PCR amplification for samples purified by the method proposed by us was about 100%. Moreover, there were almost no DNA losses during purification (loaded and eluted volumes were equal). Other advantages on this method are its rapidity and simplicity.

Bovine albumin added to the reaction mixture at a final concentration of 160 μg/ml improves the specificity of PCR amplification [4]. PCR amplification was inhibited when DNA samples from highly contaminated or jean cloths underwent only the first step of purification. In these cases the addition of albumin (160 μg/ml) had no effect [4]. On the other hand, in samples obtained after the second purification step the addition of albumin significantly stabilized or even increased PCR amplification. Noteworthy, albumin at a required concentration was added directly to the reaction mixture and not to the Taq polymerase buffer (×10) (Fig. 1, *3*).

In this work we were faced with a problem that the use of specific oligonucleotide primers for polymorphous apoB and D1S30 loci sometimes yielded unstable results and the sensitivity of PCR amplification was limited at lower DNA concentrations in the sample (<10 ng). To solve this problem we lengthened the primers: to 24 and 26 bp for apoB locus and to 29 bp for D1S30. The annealing temperatures during PCR amplification were also changed with the optimum at 62°C and 64-66°C for apoB and D1S30, respectively. The higher temperature increases the specificity of primer hybridization with target DNA sequences. This plays a crucial role for samples containing low DNA concentration and for degraded or contaminated with in-

hibitors DNA samples (Fig. 1). The sensitivity of synthesized primers is sufficient for successful PCR amplification with 2-4 ng of DNA in the reaction mixture.

Thus, the two problems were solved in the work: first, a reliable method of DNA isolation and purification from highly contaminated sources was developed and second, the length and sequences of oligonucleotide primers were determined and the conditions for apoB and D1S30 DNA amplification were optimized that allowed to perform PCR with 2-4 ng of DNA in the reaction mixture.

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