IDENTIFICATION OF HUMAN REMAINS FROM MASS GRAVES FOUND IN CROATIA AND BOSNIA AND HERZEGOVINA

Dragan Primorac, MD, Ph.D.

Laboratory for Clinical and Forensic Genetics, Spinciceva 1, 21 000 Split, Croatia.

ABSTRACT

A variety of methods have been used in our laboratory in an effort to improve identification methods. The first DNA identification we successfully performed was in 1994 when we were able to identify 5 persons found in the Kupres mass grave (Bosnia and Herzegovina). In that particular case DNA was isolated using phenol/chloroform extraction followed by Centricon 100 purification and concentration and amplified with the AmpliType PM + DQA1 system. Since that first identification different extraction methods have been used in an effort to obtain more efficient amplification. Recently, we introduced the AmpF/STR Profiler™ PCR System that allows multiplex amplification of nine STR loci (D3S1358, vWA, FGA, TH01, TPOX, CSF1PO, D5S818, D13S317, D7S820) and the amelogenin (AMEL) sex-typing locus. After analysis of more than 140 samples we noticed that the AmpF/STR™ Profiler amplification reaction for the DNA isolated from the skeletal remains is significantly more efficient than amplification by the AmpliType PM + DQA1 system. Furthermore, we observed that DNA extracted from teeth gives much better results than DNA extracted from long bones. However, in a few cases where genomic DNA was extremely degraded we formed a collaboration with other laboratories and analysis of mitochondrial DNA (mtDNA) was performed.

INTRODUCTION

Different techniques are used to identify a human corpse, depending on the circumstances and the state of remains. The most common methods utilized today include: identification of the remains by a living person who knew the deceased victim by direct facial recognition or recognition of special features such as scars or marks (tattoos), matching of fingerprints (if pre-mortem inked prints are available), dentition (if premortem dental records are available) and DNA analysis.

From 1992 through 1999, the postmortem remains of 382 persons who were killed and interred in 16 mass graves in Bosnia and Herzegovina were transported to the Department of Pathology and Forensic Medicine. The remains from most mass graves were excavated in collaboration with senior representatives in Bosnia, International observers, European observers, Physicians for Human Rights and the Committee for Missing and Imprisoned Persons.

All remains transported to the University Hospital Split, Department of Pathology and Forensic Sciences were in an advanced state of decomposition, and the majority were only skeletal remains (1). In every case, forensic examiners performed a detailed examination of the clothing and belongings of the dead, described special features, analyzed skeletal remains to estimate sex and height, and compared premortem dental records with postmortem dental records. In addition X-ray comparisons were performed for bone morphology as well as the superimposition of skull and photographic images. Unfortunately, for 30-35% of all victims the standard forensic identification methods were not sufficient and DNA identification was requested.

DNA was isolated from the bones and teeth samples in a form suitable for PCR analysis. At the beginning of our work we employed the PM and DQA1 identification kit, but after analysis of more than 90 samples it became obvious that this system worked for only 20-25% cases (2). The most common problems we faced while working with PM and DQA1 were either amplification difficulties or nonspecific hybridization that caused ubiquitous dots. Recently, we introduced the AmpF/STR® Profiler™ PCR Amplification Kit and so far more than 70 bone and teeth samples were collected and analyzed. The AmpF/STR® Profiler™ PCR Amplification Kit allowed multiplex amplification of nine STR loci and the amelogenin (AMEL) sex-typing gene. In this kit one of each locus specific primer possessed a single fluorescent dye molecule, covalently bound to 5' end that allowed laser fluorescence detection.

Furthermore, we have introduced some modifications to an original DNA isolation procedure as well as a more vigorous DNA purification. Using STR analysis the efficiency of identification by genomic DNA increases up to 85%.

MATERIAL AND METHODS

DNA Extraction:

Samples of long bones and teeth were collected for DNA analysis at the time of autopsies. All bones were cleaned from the remnant soft tissue as well as from all soil traces. Additionally, the bone surfaces were washed in warm water with a soft detergent and a brush. Subsequent to cleaning, the bones were rinsed several times with distilled water and left out until they were dry.

Once the bones were dry, 2 - 5 gr. of the material were collected for the DNA analysis. The external and internal surfaces of the bone specimens were removed by linear sawing (2-3 mm in. deep) with K9 Foot control unit, type 900 (KaVo Elektrotechnisches Werk. Vertriebsgesellschaft mbH, Leutkirch, Germany). The samples were cleaned from sawdust with the cotton presoaked in 5% commercial bleach and then, once more with the standard dental carbon brushes. It is important to underline that the sawing time (contact with the bone) should be limited to up to 3 second since the longer exposure may cause rapid DNA damage.

Separated bone fragments were washed twice in a 50 ml conical tube filed with 5% commercial bleach, following washing in a deionized water (twice) as well as in the 80% EtOH (twice). All samples were allowed to air dry for at least 3- 4 hours. The samples were placed in the steel-plated chambers, crushed with hammer and pulverized into a fine powder with the presence of liquid nitrogen. The powder was poured into a clean 15 ml polypropylene tube and 3 ml of extraction buffer (10mM Tris, pH 8.0; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS) and 100 µl 20 mg/ml Proteinase K were added to the specimens as was described earlier (3). The mix was incubated overnight at 56°C. After phenol/chloroform extraction (buffered phenol-chloroform-isoamyl alcohol solution), we performed extraction with chloroform as well. Three ml of n-Butanol was used to gain the lower aqueous layer that was transferred to the corresponding Centricon-100 concentrators. All samples were washed 3- 5 times with 1 ml of TE, concentrated and 20- 30 µl of the filtrate was obtained. Following the concentration step the samples were quantitated by mini-gel electrophoresis or by spectrometry. In most cases 1 µl of the filtrate obtained after DNA extraction was used for the PCR amplification.

DNA Amplification and Typing:

Typing was carried out using the following commercially available supplies: Amplitype PM + DQA1 amplification and typing kit, AmpliFLP D1S80 PCR Amplification Kit and AmpF/STR® Profiler™ amplification and typing kit (Perkin Elmer).

The amplifications were performed using a Gene Amp PCR system 9600 thermocycler (PE Applied Biosystems) using the original manufacturer's instructions. Amplification products were visualized in 1% agarose gels containing ethidium bromide.

The unit employed to evaluate the STR amplicons was the 310 Gene Analyser (PE Applied Biosystems). Amplified STR products were prepared for capillary electrophoresis, as were allelic ladder samples by mixing 25 µl of formamide/Gene Scan-350 (ROX) solution with 1.5 µl of PCR product. Genotyper® 2.0 software is used to convert allele sizes obtained from GeneScan® Analysis software into allele designations automatically and to build tables containing the genotype information.

NaOH Repurification:

Although the procedure published by Bourke at al. was developed to overcome potential inhibitors of Taq Polymerase while working with AmpliType PM + DQA1 and D1S80 systems we applied this procedure for the DNA extracted from the bone samples that failed to amplify with AmpF/STR® Profiler™ system (4). The filtrate gain after concentration with a Centricon-100 concentrator was placed into a Microcon-100 unit along with 200 µl of 0.4N NaOH. The final volume was reduced to 5 µl by centrifugation at 500 x g. The chamber was refilled with 400 µl of O.4N NaOH and centrifuged as described. This step was repeated once. The sample was neutralized by washing with 400 µl of 10 mM Tris (7.5) and recovered in 15 µl of 10 mM Tris (7.5).

RESULTS

The results of our study indicate the following:

1. When we compared our previous work when we used DQA1 + PM amplification kit as well as AmpFLP D1S80 PCR Amplification Kit (by that method only 25 % of mass graves victims that failed to be identify by standard forensic methods were identified) with STR analysis it became obvious that STR analysis is the method of the choice.

2. After modification of the standard DNA extraction procedure, after attempts to do re-purification of extracted DNA (with NaOH) and after introducing AmpF/STR® Profiler™ kit the percentage of identification increased to 85%.

3. Teeth samples gave us 20- 30% enhanced results when compared with results obtained from the long bones. However, DNA extracted from the teeth amplified better if extracted from the lower half of tooth (including the root of the tooth) rather than from the crown compartment.

4. During the analysis of 9 loci analyzed by an AmpF/STR™ Profiler™ PCR Amplification Kit we noticed that the D3S1358 loci in 50% of cases amplified more sites than the 2 targeted alleles when the PCR was performed prior to re purification.

5. D5S818, D13S317 and D7S820 loci (primers labeled with NED dye) are usually more difficult to analyze than loci for which primers are labeled with either 5-FAM or JOE dye.

6. Although it has been recommended by the manufacturer that 1.0 –2.5 ng of DNA is required for successful amplification we observed that approximately 20-30 ng of DNA is necessary when amplifying older bone samples.

DISCUSSION

Teeth and bones have been shown to be valuable sources of DNA evidence. Dental enamel, the hardest substance in the human body, protects the DNA rich pulp and dentin and therefore ensures a good quality of isolated DNA. It appears that the most important variables upon which identification by DNA depends are: the extent of time after death, the type of soil wherein the bodies were buried and the method of DNA extraction. For identification purposes, several population studies were performed in our laboratory and one in the neighborly laboratory (5,6,7).

The problems which forensic scientists most often face while working with DNA extracted from different samples are either DNA degradation or DNA contamination. High temperatures, salt, water or soil conditions can damage genomic DNA and therefore make the identification process extremely difficult.
The genetic analysis of those killed and buried in the mass graves through DNA from remains of bone and teeth requires the use of short sized loci that can be amplified by polymerase chain reaction (PCR) for which the short tandem repeat (STR) loci are most suitable (8). Short tandem repeats markers are polymorphic DNA loci at non-coding regions of the gene containing a repeated nucleotide sequence. Usually STR repeat units are from two to seven nucleotides in length.

During our work we did confirm findings published earlier that DNA could be extracted and amplified without decalcification (9). Also, we observed that the PCR reaction did not work or worked with low efficiency if an NaOH purification procedure was not performed. Although this particular procedure was developed for a DQA1 and PM systems we decided to test it with AmpF/STR® Profiler™ PCR Amplification Kit and we have observed that treatment with NaOH permitting amplification of many samples that originally failed to amplify.

In most of our cases the DNA extracted from bone and teeth samples had a ratio at 260/280 of between 1.5-1.6 with 1µl of the filtrated DNA giving between 0.03 – 0.065 ?g/µl. The amount of DNA extracted from the bone we used for amplification varied from 30 to 60 ng. However, DNA extracted from the teeth amplified much better even the amount used for amplification did not exceed 10 ng. Over 85 % of all samples were amplified in all 9 loci and the amelogenin (AMEL) sex-typing locus, while 15% were amplified in 8 or less loci. In cases with extensive PCR inhibition the NaOH procedure was useful in approximately 30% of the cases where STRs analysis were chosen as a method.

Furthermore, the Chelex method of DNA extraction has been used for blood and bone samples. Although this procedure is simple, rapid and involves no organic solvents, it was not suitable for bone analysis. The Chelex extraction method, however, was the method of choice for efficient DNA amplification of relatives' blood.

Currently we are one of the two DNA forensic laboratories in an area in which war victims from Croatia and Bosnia and Herzegovina totaled more than 150 000 between 1991-1992. We are in the process of testing new methods such as mitochondrial DNA typing for identification of human remains. During our 5 years period we have enjoyed a successful collaboration with the Department of Forensic medicine in Zagreb, Forensic Science Laboratory at FBI Academy, the Connecticut State Police, the New York State Police, Analytical Genetics Testing Center, and the Armed Forces DNA Identification Laboratory.

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