

TECHNICAL NOTE

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Effects of Processing Techniques on the Forensic DNA Analysis of Human Skeletal Remains*

ABSTRACT: Human remains processed by forensic anthropologists may potentially be used for genetic analysis. Therefore, the condition of the deoxyribonucleic acid (DNA) in processed remains may become an issue for future analysis. Processing techniques employed by anthropologists are highly variable and scanning electron microscopy reveals significant alterations to the bone surface depending upon the technique used. Such damage to the bone indicates differences may exist in quality and quantity of DNA extracted. This study assessed how five processing procedures used by major forensic anthropology laboratories around the country affects the amounts of DNA extracted from human rib bones and the subsequent DNA analysis. The DNA was analyzed using the short tandem repeat (STR) locus CSF1PO and amelogenin. The findings indicate processing procedures used by forensic anthropologists do not adversely affect DNA analysis but prolonged exposure to heat during processing may decrease the yield of information from the DNA.

KEYWORDS: forensic science, forensic anthropology, deoxyribonucleic acid (DNA) typing, polymerase chain reaction (PCR), short tandem repeat (STR), CSF1PO, amelogenin, skeletal preparation, bone

When forensic investigators are confronted with human remains that are buried, in advanced stages of decomposition, or even skeletonized, they may enlist the expertise of a forensic anthropologist. The forensic anthropologist utilizes investigative techniques that detail skeletal and dental features as means to establish a potential identification. When such conventional methods for human identification cannot produce a positive identification, deoxyribonucleic acid (DNA) typing may provide a further avenue of investigation. In such cases, the DNA analyst is usually presented with a bone that has already been cleaned and analyzed by the forensic anthropologist. To date, no systematic investigation has determined if any of the common processing techniques used by forensic anthropologists to remove soft tissue from human remains affects the subsequent DNA analysis.

Forensic DNA analysts routinely encounter samples containing degraded DNA. The period of time required for degradation of DNA depends on the physical and chemical nature of the surrounding environment (1). *In vivo* DNA degradation is due to processes of oxidation and hydrolysis; however, specific DNA repair mechanisms counteract this *in vivo* damage. After death, nucleic acids undergo spontaneous degradation and remain unrepaired because

these protective mechanisms are no longer present. In addition, the DNA is also susceptible to derivative processes due to environmental variables such as temperature, chemical exposure, and biological activity (1–5).

Degraded DNA, whether from a modern forensic or ancient/archeological source, can adversely affect DNA analysis. Artifacts generated during the polymerase chain reaction (PCR) process, attributed to the degraded state of DNA, have been reported to reduce the reproducibility of the DNA analysis or produce incomplete STR profiles because of allelic dropout, particularly among larger STR loci, due to stochastic effects (1,4,6,7). In addition, inhibitors co-extracting with the DNA that are inherent to bone, or introduced by the environment (i.e., fulvic acids), further complicate PCR (1,2). Thus, it is important to understand the effects various environmental exposures and manipulations to biological materials (i.e., bone) may have on the potential use of DNA in the identification of unknown skeletal remains.

When an anthropologist examines skeletal remains, it is usually necessary to remove any of the remaining soft tissue. There are a variety of techniques employed by forensic anthropologists to facilitate the removal of soft tissue (8–14). Several methods routinely used have been investigated for their effects on the structural integrity of bone (18–20). Brittleness, chalkiness, weight loss, matrix degradation, and surficial changes to bone visualized using scanning electron microscopy (SEM) have been observed and reported for bones exposed to various cleaning procedures (10,12,13,16–21). The destruction of bone at the microscopic level could be problematic for DNA analysts because a correlation exists between microscopic morphological preservation of bone and the recovery of DNA (22).

It is unknown what effects these different techniques of soft tissue removal may have on the subsequent DNA analysis. The purpose of this study was to investigate whether commonly used processing

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techniques could adversely affect the subsequent use of the bone specimen for DNA analysis. Investigations reported here indicate that the commonly used methods for treatment of skeletonized or partially skeletonized remains do not in general adversely affect subsequent DNA analysis. Methods involving prolonged heating appear to produce some damage that may interfere with subsequent DNA analysis. Despite some minor difficulties, common short tandem repeat (STR) based DNA analyses were successful on most samples.

Methods

Bone Samples

The samples used in this study are from the William M. Bass Donated Skeletal Collection at the University of Tennessee-Knoxville. This collection is comprised of human skeletal remains donated for scientific research. The Institutional Review Board of the University of Tennessee Medical Center approved the study.

Six ribs were removed from each of two individuals, for a total of 12 samples. Individuals 09-00 (female) and 13-00 (male) were placed in the outdoor Anthropological Research Facility, nine months and five months respectively, prior to collection of the rib samples. The use of rib bones from single individuals was proposed for the following reasons:

- DNA densities vary from individual to individual. Comparisons of the same bone type from the same individual will create less background variation in results.
- Each individual will have different DNA profiles. The same DNA pattern is needed for comparisons across methods to detect their effects on subsequent DNA analysis (i.e., allelic dropout).
- Both male and female were selected to permit the monitoring of amelogenin alleles from both X- and Y-chromosomes.
- Each of the bones tested should be of the same time since death to ensure variation observed is attributable to processing techniques rather than degradation due to differential exposure to the environment.
- Multiple bones of similar shape and size from a single individual allows for the examination of multiple processing techniques, while one bone can serve as a control. Therefore, the bone is not sampled prior to processing, which compromises the integrity of the bone. This eliminates the question of whether or not observed variation among techniques is attributed to the technique itself, the compromised integrity of the bone, or a combination of both.

Processing Techniques

The techniques used for bone processing were solicited from major forensic anthropology laboratories within the United States. Five different techniques (B-F) were chosen for assessment in this study due to sample size. These five techniques are representative of most of the processing techniques in use and were thus selected for testing on the 12 sampled ribs.

The ribs were processed at the Regional Forensic Center at the University of Tennessee Medical Center. The processing procedures were carried out in large metal pots heated by hotplates. All equipment used for processing was thoroughly cleaned with hot soapy water and rinsed extensively between uses. All samples were rinsed under lukewarm water, scrubbed with a plastic bristle brush, and dried overnight at room temperature after the conclu-

sion of the processing treatment. Processing techniques for six ribs:

- A: No treatment (control).
- B: Simmer (for the purpose of this paper, simmer is defined as a few bubbles that form slowly and burst just before they reach the surface) in 4 L of water for 1.5 h.
- C: Simmer in 4 L of water containing $\frac{1}{2}$ cup of ALCONOX[®] for 1.5 h.
- D: Simmer in 4 L of water containing $\frac{1}{2}$ cup of Arm & Hammer[®] Super Washing Soda powder for 1.5 h.
- E: Boil (for the purpose of this paper, boil is defined as the formation of bubbles that rise in a steady pattern and burst once they reach the surface) in 4 L of water for 1.5 h, scrub with a plastic bristle brush and air dry overnight (day one). On day two, boil in water containing $\frac{1}{2}$ cup of borax powder for 1.5 h. The pot was removed from heat and allowed to sit at room temperature overnight. This boiling/cooling process was repeated for a total of six times using the same water solution. On the seventh day, the rib was removed and placed in a fresh borax solution and simmered for 1.5 h.
- F: Simmer in 4 L of water containing $\frac{1}{2}$ cup of ALCONOX[®] for 1.5 h on day one, allow to cool overnight. On day two, simmer for 1.5 h in water containing $\frac{2}{3}$ cup of TSP (a concentrated degreaser).

Bone Sampling

The workstation and all cutting tools were thoroughly cleaned with a freshly prepared 10% bleach solution followed by 100% ethanol. Bone samples were removed from the superior border of the rib, approximately 5 cm lateral of the tubercle, using a CRAFTSMAN[®] Rotary Tool. Prior to cutting the bone, the area of interest was wiped with 10% bleach, rinsed with reverse osmosis water, and sanded to remove the outer layer to avoid contamination from previous handling of the rib. Approximately 5 cm of bone, weighing 1.1–1.5 g, was removed from each rib and stored in ultraviolet (UV) irradiated 15 mL polypropylene tubes.

Contamination Precautions

The polymerase chain reaction (PCR) is sensitive to low levels of DNA. As a result, it is important to implement precautions to avoid the inadvertent introduction of extraneous DNA. Such an event can ultimately compromise the accuracy of the DNA typing results. Stringent contamination precautions, similar to those used in ancient DNA (aDNA) analysis, were taken to avoid such an occurrence (23). Disposable gowns, gloves, hairnets, and facial masks were worn whenever handling a sample and while performing pre- and post-PCR procedures. Work surfaces and equipment were cleaned with 10% bleach and 100% ethanol and, when possible, were UV irradiated.

All pre- and post-extraction and pre- and post-PCR activities were performed in separate rooms with dedicated equipment. Preparation of buffers and other reagents, bone grinding, and DNA extraction were performed in laminar flow hoods equipped with UV bulbs. The laminar flow hoods were treated with 10% bleach and ethanol and UV irradiated before each use. All reagents were aliquoted into single use containers that had been UV irradiated. Aerosol resistant tips were used at all times. Polymerase chain reaction (PCR) was set up in a room dedicated for this sole purpose, and DNA separation (slab gel and fragment analysis) was carried out in a third room.

DNA Extraction

Bone fragments were rinsed with 10% bleach followed by a thorough rinsing with reverse osmosis water. Subsequently, the fragments were UV irradiated on each side for 15 min, for a total of 30 min of UV exposure. The bone fragments were sealed in plastic bags, placed in liquid nitrogen for 5 min, and then ground in a coffee grinder. The ground bone was transferred to a new 15 mL tube and stored at room temperature until extraction.

The DNA was extracted from ground bone samples using a salting-out technique described by Cattaneo et al. (24). An extraction blank was always included to detect contamination. This method uses proteinase K digestion followed by a differential precipitation of DNA using high salt and ethanol. Three milliliters of White Cell Lysis buffer (10 mM Tris-HCl, pH 7.6, 10 mM sodium EDTA, 50 mM NaCl) and 25 μ L of proteinase K (20 mg/ml) were added to each ground specimen and the specimens were incubated overnight at 42°C. The following day the temperature of the incubator was increased to 75°C for 15 min to deactivate the proteinase K. One milliliter of saturated sodium acetate was added and the tube was shaken manually for 30 s and centrifuged at 4000 \times g for 10 min. The supernatant was transferred to a new 15 mL polypropylene tube and 4 mL of 100% ethanol was added, the sample was gently mixed for 10 min through periodic inversions and centrifuged at 4000 \times g for 10 min. The supernatant was discarded and the pelleted DNA was resuspended in 250 μ L of cold 70% ethanol and transferred to a 1.5 mL microcentrifuge tube and centrifuged at 13 000 \times g for 10 min. The resulting DNA pellet was air dried for 1 h at room temperature and resuspended in 100 μ L of silica-purified molecular grade water and stored at -20°C until analysis.

A 50 μ L portion of the DNA sample was subjected to additional purification with a silica-based matrix using the GENE CLEAN II Kit (Bio 101, Vista, CA). The sample was treated following the manufacturer's instructions and reconstituted in 50 μ L of silica-treated molecular grade water (25).

PCR

Samples were amplified per manufacturer's instructions using a GENEAMP[®] PCR 2400 (PE Applied Biosystems, Foster City, CA) using the monoplex GenePrint[®] Fluorescent STR System (Promega Corp, Madison, WI) primers for the STR locus CSF1PO and amelogenin (26). The two loci were amplified separately using AmpliTaq Gold[®] DNA polymerase (PE Applied Biosystems, Foster City, CA) and Gold ST[®]R buffer (Promega Corp, Madison, WI). A PCR blank containing all reagents and no DNA was included.

The 25 μ L reaction contained 17.45 μ L of silica-purified molecular grade water, 2.5 μ L of the locus-specific 10X primer pair, 2.5 μ L buffer, 0.25U polymerase, and 2.5 μ L of sample. The amplification protocol was a modified version of protocol number 12 supplied with the GenePrint system (26).

- 95°C for 11 min, 96°C for 2 min
- 10 cycles of 94°C for 1 min, 60°C for 1 min, 70°C for 1.5 min
- 32 cycles of 90°C for 1 min, 60°C for 1 min, 70°C for 1.5 min
- 60°C for 30 min and hold at 4°C

Amplified samples were subsequently purified for fragment analysis using the QIAquick PCR Purification kit (QIAGEN Inc. Valencia, CA) per manufacturer's instructions (27).

Fragment Analysis

DNA products were screened for successful amplification by gel electrophoresis on 10% polyacrylamide gels. Successfully amplified products were then analyzed by capillary electrophoresis on an ABI Prism[®] 310 Genetic Analyzer according to the manufacturer's specifications using 1 μ L of amplified sample (26).

Results and Discussion

DNA typing, also referred to as DNA fingerprinting, has become an integral component of forensic investigation. STR analyses have usurped previous DNA typing systems because the STR system is PCR based, can work with low-quantities of DNA, offers high power of discrimination, and loci analyzed are small in size (100–400 bp) (6). The small target size makes STR markers ideal for analyzing degraded samples typically encountered in forensic cases. In addition, less DNA is required for analysis than sequential systems because STR systems permit multiplexing of reactions. In the United States, 13 STR loci, as well as the amelogenin locus (a sex-typing marker), are routinely used within the forensic genetic community for identification of individuals. Recently, a 16 locus multiplex system containing two additional STR loci was validated (28). In this investigation, two of these loci were used as representatives of the commonly used loci. The CSF1PO locus was chosen because it is one of the larger STRs, producing amplicons that range from 291 bp–331 bp. Therefore, it would be more sensitive to degradation during processing which may result in allele dropout. The amelogenin locus was also chosen because sex determination is an important aspect of forensic analysis. In addition, this locus produces a relatively small PCR fragment (X = 212 bp, Y = 218 bp) and therefore might be less sensitive to potential processing related effects.

The genotypes of the samples were determined by comparing the electrophoretically separated PCR product fragments against an allelic ladder of the specific locus using the Genotyper software program. All of the 09-00 samples were heterozygous for the CSF1PO locus with fragments of 316 bp and 320 bp in length and all were correctly assigned female based on the presence of the amelogenin X-chromosome specific fragment. The 316 bp and 320 bp fragments at the CSF1PO locus did not correspond to the genotype of the female investigator who processed the samples. All of the 13-00 samples were homozygous for the CSF1PO locus with a single fragment of 316 bp in length and all were properly typed as male based on the presence of both the X- and Y-chromosome associated amelogenin fragments. The peak heights for all fragments for all specimens were above 150 relative fluorescence units (RFUs), a height generally used in forensic laboratories to determine if the analysis was acceptable.

The samples were extracted from ground bone using the salting-out technique because Cattaneo et al. (24) reported that this method provided similar results to the established phenol-chloroform extraction technique. The salting out technique was preferred because it avoided the use of hazardous chemicals. Mechanical reduction of the bone sample was used in order to increase the surface area in contact with the extraction buffers and enzymes, and was chosen because it was the method implemented by Cattaneo et al. (24). An alternative method involves the chemical reduction of the bone sample by decalcifying the bone with a series of incubations in EDTA. However, both methods of sample reduction provide adequate DNA template amenable to amplification using PCR (29).

Evidently, the salting-out technique does not always efficiently remove inhibitors because additional purification was necessary to achieve successful DNA amplification. The results of DNA samples

TABLE 1—Record of successful amplifications.

Sample	Non-Geneclean II Amel/CSF1PO	Geneclean II Amel/CSF1PO
09-00A	-/-	-/-
09-00B	-/-	+/+
09-00C	-/-	+/+
09-00D	-/-	+/+
09-00E	-/-	+/+
09-00F	-/-	+/+
13-00A	+/-	/+
13-00B	+/-	/+
13-00C	+/-	/+
13-00D	-/-	+/+
13-00E	-/-	-/-
13-00F	+/+	

Amel = Amelogenin, - = Not Successfully Amplified, + = Successfully Amplified, Blank = Not Attempted.

that were assayed with and without secondary purification using the GENECLEAN II Kit (Bio 101, Vista, CA) are summarized in Table 1. While some samples produced analyzable fragments without additional purification, the best results were obtained for samples that had been purified of small inhibitory molecules using the silica-based purification system.

Two samples, 09-00A and 13-00E, did not produce analyzable products despite changes in sample amount, annealing temperature, dilutions, or other PCR parameter manipulations (not shown). The failed amplification of sample 09-00A seems counterintuitive, because this sample was not processed in any way. A possible explanation for the failure to amplify the 09-00A sample include the potential introduction of additional inhibitors associated with the presence of hemoglobin (as indicated by red/brown coloration) or other biological material in the inner cavity of all the ribs from that individual. This red/brown coloration in the inner cavity of the rib diminished with processing. It was not unexpected that amplifications for sample 13-00E were unsuccessful for both loci because the prolonged exposure to high temperature may have further fragmented the DNA. On the other hand, the successful amplification of sample 09-00E may be attributable to a decrease in the aforementioned inhibitor or other inter-specimen variation. While the theories above may be correct, there is insufficient data to validate them at the present time.

Two quantification methods were investigated: PicoGreen® (Molecular Probes, Eugene, OR) and QuantiBlot Human DNA Quantification Kit (PE Biosystems, Foster City, CA). However, results of the DNA quantification analyses were not presented because neither technique was successful in the quantification of extracted DNA. However, it has been demonstrated that samples can be successfully amplified even though a quantification system fails to detect any human DNA (30). Even if one or both of these quantification techniques had provided results indicating the amount of DNA extracted from each sample, this information would be of little value since neither technique can differentiate between amounts of total DNA and amounts of useable DNA (i.e., available for PCR detection).

All of the samples were successfully amplified, except for 09-00A and 13-00E. This finding suggests that the use of a processing technique may reduce some inhibitors present in the bone. In addition, over-exposure to heat appears to adversely affect the subsequent DNA analysis. However, with this small sample size, the reasons for the differences in the successful amplification between samples 09-00A/13-00A and 09-00E/13-00E cannot be clearly evaluated.

Conclusion

The purpose of this study was to determine if the techniques commonly employed by forensic anthropologists to process human remains affects the subsequent DNA analysis. This was accomplished using processing techniques solicited from major forensic anthropology laboratories and performing DNA analysis with techniques employed by the forensic genetic community. Results presented here suggest that the chemicals used in processing techniques to remove soft tissue do not appear to affect the subsequent DNA analysis. There is preliminary indication that exposure to excess heat during processing may contribute to the decreased ability to amplify the larger STR locus. Previous reports of the effects of excess heat on the integrity of the bone itself (3–9) support this potential interference. Based on the information available, a ranking of the processing techniques from satisfactory to unsatisfactory based on how easily assayable product was obtained would be C, D, F, B, A, and E. Regardless of which technique is employed, the length of time to process remains is dependent upon the condition of the remains as well as the amount of soft tissue present and should be taken into consideration to prevent the over-processing of remains. In addition, exposure to extreme heat, more specifically boiling, should be kept at a minimum or preferably avoided altogether.

Thousands of bone and tissue fragments have been processed for DNA analysis in multiple laboratories over the years, so there is much anecdotal information available regarding the effects of uncontrolled exposures to heat and chemicals on DNA analysis. However, a systematic evaluation of the controlled processing of bone samples for DNA analysis can provide a guideline for future work when a choice of processing techniques is available. While an avoidance of excessive time and temperature is suggested, it may be advantageous to include at least a minimum amount of simmering to assist in the removal of soluble inhibitors of the subsequent amplification process. In addition, it is recommended that silica or some other matrix-based secondary purification step be added to the salting-out extraction procedure to improve success in obtaining assayable PCR products.

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