TECHNICAL NOTE

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A Silica-Based Mitochondrial DNA Extraction Method Applied to Forensic Hair Shafts and Teeth*

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ABSTRACT: The purpose of this study is to evaluate the applicability of a nonorganic DNA extraction method for use in the analysis of environmentally compromised forensic hair shaft and tooth samples. The condition of the samples included cases of water decomposition, severe incineration, and varying stages of putrefaction. Enzymatic amplification and manual sequencing of the first segment of the mitochondrial hypervariable region were performed successfully on each of the 20 autopsied individuals. The results indicate that the silica-based extraction method produces mtDNA suitable for genetic identification from forensic samples including hair shafts and teeth.

KEYWORDS: forensic science, mitochondrial DNA, DNA sequencing, hair shafts, teeth, DNA typing, polymerase chain reaction, environmental effects

Human mitochondrial DNA (mtDNA) is a maternally transmitted circular genome approximately 16 569 bp in length (1). The most polymorphic region of the human mtDNA genome is concentrated in two hypervariable segments within the noncoding, D-loop region (2). The mutation rate in this area is 5 to 10 times higher than that of nuclear DNA (3,4). The high mutation rate of mtDNA in this area combined with the maternal mode of transmission allows comparison of mtDNA sequences between maternally related individuals, and is routinely employed for identification purposes (5–13). In addition, mtDNA is present in hundreds to thousands of copies per cell and therefore may be better suited than nuclear DNA for ascertaining genetic information in cases where DNA amounts may be limited, degraded or both.

It is important to understand the effect of environmental factors on the quality and quantity of DNA obtained from forensic samples. The effect of adverse environmental parameters on the isolation of DNA suitable for identification from teeth has been reported (6,11,14–16). Laboratory and individual case studies have shown that teeth provide a well-protected source of DNA. Unlike teeth, hair is not well protected; however, hair samples are more easily collected and may be available in cases where teeth are not present. Hair is the most common biological forensic evidence found at a crime scene, making hair shafts a potentially valuable source of DNA for forensic analysis.

Until recently, molecular techniques restricted hair studies to the DNA extracted from the hair bulb (7,17,18); however, new methods have been reported for the extraction of DNA from modern hair shafts (5,13,19–22). Uchihi et al. (19) explored the amount of hair shaft needed to perform successful PCR amplifications of HLA-DQA1 without inhibiting the reaction with too much melanin. Yoshii et al. (21) examined the effects of natural and artificial hair color as well as sampling distance from hair root on the ability to sufficiently analyze mtDNA. Wilson et al. (20) report the successful sequencing of mtDNA, and in another study they investigate the effects of various contaminants on the ability to extract and amplify mtDNA from hair shaft samples (13). Allen et al. (5) reported the successful use of shed hair as a source of mtDNA for linking a suspect to a crime scene. An extraction method for ancient mtDNA from hair shafts is described by Baker (22). However, hair as a source of mtDNA for the identification of deceased individuals exposed to environmental conditions has not been reported.

Previous analyses of mtDNA from hair shafts have reported the use of organic solvents, usually phenol/chloroform, in the extraction methodology (13,19,20). Analysis of DNA from ancient and historical anthropological samples has employed a silica-based method for DNA extraction (23). Our laboratory's successful use of this methodology on ancient specimens led us to investigate the utility of the silica-based method for forensic hair and teeth. We report the successful application of a silica-based DNA extraction to a panel of forensic hair and teeth samples obtained from autopsied individuals.

Materials and Methods

Samples

Tooth ($n = 15$) and hair ($n = 19$) samples were collected during autopsy by Dr. William F. McCormick, MD, Deputy Chief Medical Examiner, State of Tennessee, and stored at room temperature for 1 to 3 years before the DNA was extracted. The Institutional Review Board of the University of Tennessee approved the collection of samples. Table 1 gives a summary of the characteristics of each sample at the time of collection. The samples represent 20 unrelated individuals in varied stages of decomposition: putrefied ($n = 14$), burned ($n = 5$), and drowned ($n = 1$). The hair samples are cut hair shafts without the hair root and include both scalp and pu-

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TABLE 1—*Sample summary.*

						Hair	
ID	Age	Sex	Race	Condition	Tooth Type	Type	Color
1	27	M	W	<72 h	canine	scalp	brown
2	29	M	W	<72 h	premolar	scalp	brown
3	57	M	W	<72 h	premolar	scalp	gray
4	45	M	W	<72 h	premolar	scalp	gray/brown
5	80	M	W	<72 h	premolar	scalp	white
6	21	M	W	<72 h	premolar	scalp	black
7	59	M	W	<72 h	incisor	scalp	brown
8	66	M	W	<72 h	premolar	scalp	brown
9	47	M	W	<72 h	none	scalp	dark brown
10	28	M	W	>72 h	canine	scalp	brown
11	30	F	W	>72 h	molar	scalp	dyed blonde
12	62	M	W	>72 h	canine	scalp	brown/gray
13	53	M	W	>72 h	none	scalp	brown
14	35	F	W	>72 h	none	scalp	dark brown
15	35	M	W	burn	canine	scalp	black
16	44	M	B	burn	premolar	pubic	black
17	40	F	W	burn	premolar	pubic	brown
18	44	M	W	burn	molar	none	none
19	43	M	W	burn	none	scalp	brown
20	20	M	W	drown	none	scalp	brown

bic hair. Tooth specimens consist of incisors, canines, premolars, and molars.

Fourteen of the samples were in different stages of putrefaction. Nine of these cases had a time since death estimated at less than 72 h with mild to moderate putrefaction. Severe putrefaction was observed in five cases that had a time since death of greater than 72 h. There were five cases in which the individuals had undergone severe incineration. In these cases, the entire body was burned and the hair had become extremely brittle and flaky. One sample was collected from a drowning victim who was found after 96 h submerged at a depth of 6 m (20 ft).

Contamination Precautions

Stringent precautions were employed to eliminate the possibility of contamination. Filtered pipette tips were used at all times. All microcentrifuge tubes were ultraviolet (UV) irradiated for at least 5 h before use. All amplifications included blanks in which all reagents were used but no DNA was added. An extraction blank using extraction buffer exposed to all procedures was always included. To prevent possible cross contamination, tooth and hair samples were extracted on different days.

Extractions took place in a dedicated clean room in a laminar-flow hood using equipment that had been cleaned with 10% bleach, followed by 100% ethanol and UV irradiation for 24 h before each use. Polymerase chain reaction (PCR) took place in a room designated for this purpose alone, and gel electrophoresis and sequencing took place in a third room.

DNA Extraction

DNA was extracted by our modification (22) of the silica/guanidine thiocyanate (GuSCN) method described in Boom et al. (24). Extraction buffer [10 M GuSCN, 0.1 M Tris-HCl (pH 6.4), 0.2 M EDTA (pH 8.0), 1.3% Triton X-100] was prepared according to Boom et al. (24). Contaminating DNA was removed from the buffer by the addition of 1.5 g of silica, mixing thoroughly, followed by centrifugation to pellet the silica. The extraction buffer was distributed into single-use aliquots. In order to simplify the subsequent extraction procedure, GlassMilk®, from a GeneClean II Kit (Bio 101, Vista, CA) substituted for the prepared silica described by Boom et al. (24).

Hair Extraction

Two centimeters of a single strand of hair from each sample was washed according to Wilson et al. (20). Hair was then placed in a pretreated 0.2 mL glass tissue homogenizer (Kontes Glass, Vineland, NJ) and ground in 100 μL of extraction buffer. The homogenate was transfered to a UV-irradiated microcentrifuge tube and was pooled with another 100 μL of extraction buffer used to rinse the homogenizer. Samples were placed in an incubator at 60°C with slight agitation overnight (10 to 24 h). The DNA was then isolated using the GeneClean II kit. Three volumes of sodium iodide and 5 μL of GlassMilk® were added to the sample and incubated at 57°C for 15 min with slight agitation. Following incubation, the sample was centrifuged for 5 min at 12 000 × *g* and washed twice with the Bio 101 New Wash® solution. Any remaining New Wash® was removed by centrifugation for an additional 3 min. The sample was eluted in 30 μL of 10 mM Tris/1 mM EDTA buffer (pH 7.6) (TE) and incubated at 56°C for 10 min. The sample was then centrifuged for 5 min and the supernatant was removed and stored at −20°C until amplification.

Tooth Extraction

Each tooth was soaked in hydrogen peroxide for 30 min, rinsed with 10% bleach followed by water that had undergone reverse osmosis (RO water), and UV-irradiated for 30 min on each side. The tooth was then placed in a Seal-A-Meal® bag, frozen with liquid nitrogen, crushed, and placed in a UV-irradiated tube containing 1 mL of extraction buffer. Samples were placed in an incubator at 60°C with slight agitation overnight (10 to 24 h). The supernatant was transfered to another UV-irradiated microcentrifuge tube and the DNA was isolated using the GeneClean II kit. The remainder of the DNA extraction process follows precisely that of hair.

DNA Amplification

The 25 μL PCR reaction contained PCR buffer [10 mM Tris (pH 8.3)], 1.7 mM $MgCl_2$, 200 μM each dNTP, 10 pmol of each primer, 2 units of AmpliTaq Gold (PE Applied Biosystems, Foster, CA) and 1 to 2 μL of extracted DNA overlaid with sterile mineral oil. Tooth DNA was amplified for 30 cycles and hair DNA for 36 cycles in a Perkin Elmer Cetus programmable thermocycler. A 95°C, 4 min denaturation/enzyme activation step preceded the cycling steps of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min.

Two different primer sets were used to amplify fragments of 447 base pairs and 392 base pairs containing segments from the two hypervariable regions (Table 2). Region I was sequenced in all samples and Region II was sequenced in a subset of samples. The PCR product was purified with a QIAquick Purification Kit (QIAGEN, Valencia, CA) before sequencing. Cycle sequencing was performed using a Sequenase Cycle Sequencing Kit (New England BioLabs, Beverly, MA) and labeled with P^{32}. The sequencing reactions were separated on a denaturing 6% polyacrylamide sequencing gel (National Diagnostics, Atlanta, GA).

TABLE 2—*Summary of primers.*

Primer/Region	Sequence
Region I	
L16070	5'-ACCCATCAACAACCGCTATGTA-3'
H16530	5'-AAGGGGAACGTGTGGGCTATTT-3'
Region II	
L16530	5'-AAATAGCCCACACGTTCCCCTT-3'
H375	5'-TTGAAATCTGGTTAGGCTGGTG-3'

Results

A total of 15 teeth and 19 hair shafts from 20 unrelated individuals were analyzed in this study. Sequence analysis of mtDNA was successful for each of the samples. Neither the condition of the tooth nor type of tooth used (canine, molar, premolar, or incisor) made a significant difference in the quality of sequence obtained. Likewise, all of the hair samples, regardless of the environmental condition, were suitable for analysis. Neither the type of hair tested (head or pubic) nor the color of hair affected the ability to amplify or sequence the material. The single exception was an individual who had bleached blonde hair. This sample required a larger aliquot (3 μL of the 30 μL elution) of DNA to be added to the PCR reaction in order to obtain a signal equivalent to that of other samples (Lane 4 of Fig. 1).

Sequencing of the hypervariable segments identified unique sequence variations in each individual. Where both hair and tooth samples were available, the same sequence variation was detected in DNA extracted from both sources.

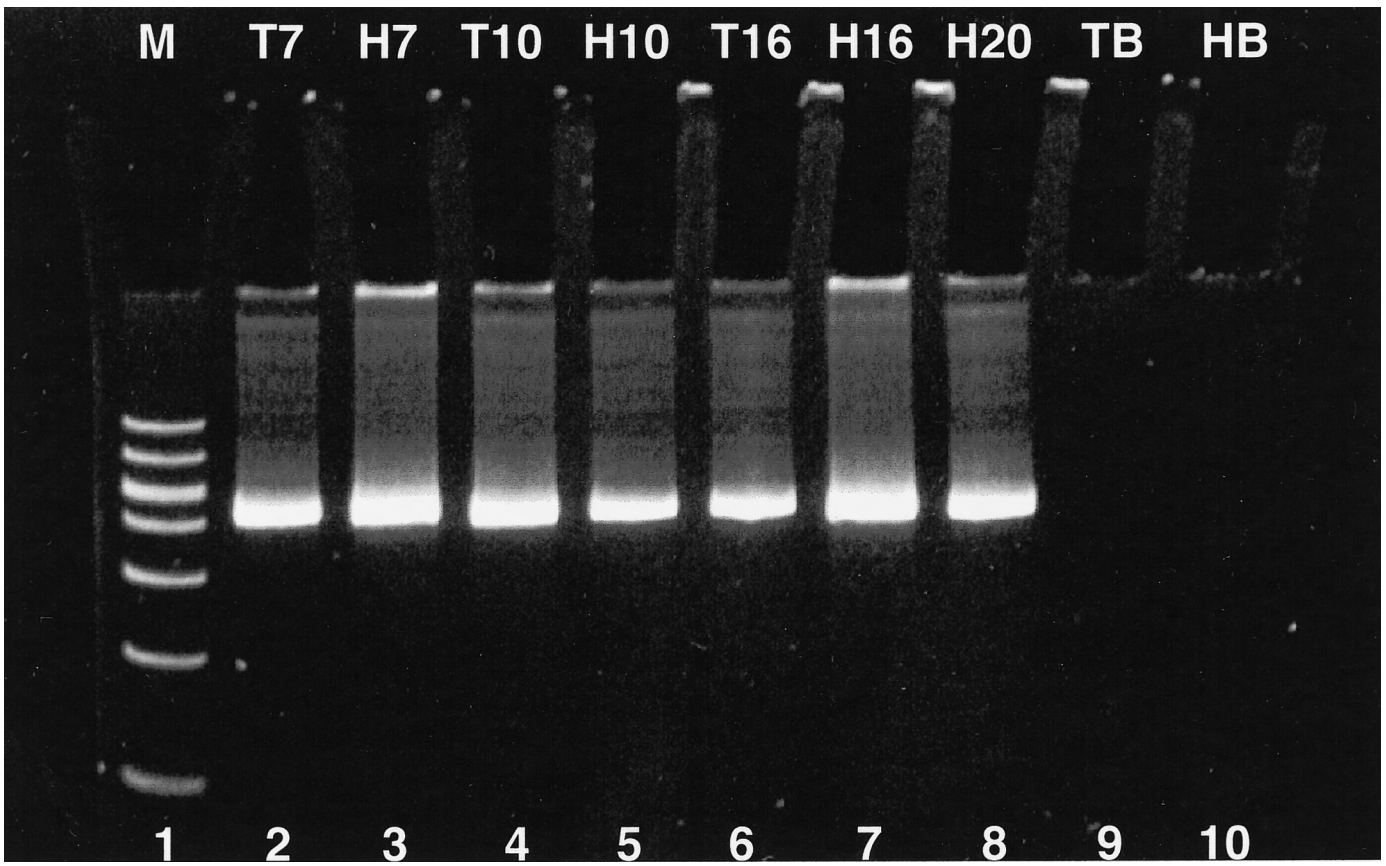

FIG. 1—*PCR products of these samples were amplified with Region I primers, electrophoresed in a 12% polyacrylamide gel and stained with ethidium bromide. Lane 1: marker (each band represents 50 ng of double-stranded DNA); Lane 2: hair #2 (<72 h); Lane 3: hair #12 (>72 h); Lane 4: hair #11 (>72 h—bleached hair); Lane 5: hair #16 (incinerated); Lane 6: hair #20 (drowned); Lane 7: extraction blank; Lane 8: PCR blank.*

Discussion

Numerous laboratory and case studies have shown that DNA can be recovered and analyzed from teeth exposed to a variety of environmental conditions (6,11,14–16). The present study provides further support for the conclusion that teeth are a reliable source for DNA even after extended time or adverse environmental stress.

In addition, these results show that hair can also reliably provide DNA for identification purposes from individuals exposed to environmental stresses, including severe incineration. Sweet et al. (11) demonstrated the utility of teeth as a source of DNA from an individual exposed to prolonged severe incineration. The multiple samples in our study exposed to incineration included one individual with a small patch of very brittle charred hair as the only remaining body hair. This hair sample (Lane 5 of Fig. 1) provided mtDNA equally as useful as that obtained from the individual's corresponding tooth sample.

The bleached hair in this study was successfully amplified on the second attempt when more extracted DNA was added for amplification. This suggests that the DNA from dyed hair may be more damaged or degraded. Yoshii et al. (21) and Wilson et al. (13) have also examined the effect of dyes on hair shaft DNA and report varied results.

Results reported here were obtained using 2 cm of a single hair. Only 1 μL of the 30 μL extract was required to produce sufficient product for subsequent sequencing. Maximization of amplicon production from hair sample DNA required a $MgCl_2$ concentration of 1.7 mM versus the 1.5 mM for tooth and control sample DNA. This difference may be due to the presence of charged molecules such as melanin that are co-extracted from the hair samples (19). Furthermore, we found that the addition of 5 μL or more of the DNA extract will completely inhibit PCR reactions.

The samples included in this study were stored a maximum of three years at room temperature. Previous forensic hair studies included samples of similar age while studies of teeth often included samples stored up to 20 years (6,15). Anthropological studies of DNA extracted from bone samples that are hundreds to thousands of years old using silica-based methods (23,25) suggest the applicability of this technique to the examination of forensic samples. Our laboratory has used this method to successfully analyze 100- to 300-year-old hair samples and 3000-year-old teeth (26,27).

Any amplified product analysis of DNA must consider the problems of contamination by either co-collected biological contaminants or contaminants within the laboratory. The analysis of mtDNA is even more sensitive to contamination due to the high copy number present in each cell. This study includes the quality controls recommended by Wilson et al. (13) as well as those routinely used in ancient DNA laboratories, and produced no evidence of contamination in any of the PCR or extraction blanks. Previous reports have suggested that contamination detected in PCR and extraction blanks at levels less than 10% of the sample signal does not prevent correct typing results (13,20); however, we believe that with enough care contamination can be eliminated.

This study provides evidence that hair shaft samples from deceased individuals can provide mtDNA useful for genetic analysis independent of many environmental conditions. The silica-based extraction method has been shown to provide a viable alternative to the commonly used organic method. In addition, the silica method may improve the yield of small to moderate length DNA fragments because it is not dependent on a DNA precipitation step and may decrease chemical damage compared with phenol/chloroform methods.

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