DENTAL CALCULUS AS AN ALTERNATE SOURCE OF MITOCHONDRIAL DNA FOR ANALYSIS OF SKELETAL REMAINS

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Mitochondrial DNA (mtDNA) is widely used in studies of affinities among living peoples and prehistoric populations represented by skeletal remains excavated at archaeological sites. Although many Indian groups see the utility of using mtDNA analysis as a means of connecting past and present, cultural norms regarding treatment of human remains prevent the use of destructive techniques in obtaining DNA. In this paper we discuss the utility of using dental calculus collected from a number of individuals comprising a pre-contact burial site (CA-SOL-357; A.D. 600-1000) as a possible source of mtDNA.

As an Okanogan and Lakes Indian affiliated with two of the 12 tribes that comprise the Confederated Tribes of the Colville Reservation and an archaeologist studying osteology, I (the senior author) find myself vacillating between these systems of belief. There are times DNA analysis may be the only method that can answer the question of ancestry. Yet the traditional method of this analysis is destructive, and therefore most American Indians consider this treatment of their ancestors unacceptable. There are substances that allow indirect analysis of DNA, for example, coprolites (Jenkins 2007) and hair (O'Rourke et al. 2000). However, when working with ancient DNA it may be difficult to ascertain the origin of the source material. Dental calculus (further called calculus) is a substance for indirect DNA analysis that can be directly linked to a specific individual. This study used calculus obtained from prehistoric skeletal remains as a source of mtDNA in an effort to find a middle ground between American Indian systems of belief and scientific methods. To my knowledge, calculus has not previously been used as a source for human mtDNA.

STUDY AREA

The calculus used in this study was removed from the teeth of human skeletons recovered from SOL-357, a prehistoric cemetery near the present-day city of Vacaville (Kerr-Siefkin 1993). The cemetery was dated using obsidian hydration methods to A.D. 600-1000 (Kerr-Siefkin 1993). Therefore mtDNA extracted from these samples should have originated from one of the four matrilines most commonly found among modern North American Indian groups.

BACKGROUND

Calculus is composed of crystallized dental plaque. Plaque consists of minerals, epithelial cells (Teymoortash et al. 2002), microbes, salivary proteins, and glycoproteins, some of which comprise the acquired enamel pellicle (Eggen and Rölla 1985; Teymoortash et al. 2002). The enamel pellicle is a biofilm composed of glycoprotein that is required for microbial attachment to the various surfaces of the

oral cavity (Marsh and Bradshaw 1995; Mergenhagen et al. 1987). Perusing DNA extraction protocols, one can see that the typical sources for DNA analysis include saliva, blood, semen, buccal cells, nail clippings, hair roots and shafts, oil from finger prints, body tissues, bones, and teeth. Calculus contains three of the sources typically used for DNA analysis: epithelial cells, saliva, and proteins.

In 1995, Kawano et al. utilized calculus as a source of DNA to determine the sex of bleached skeletal remains of unknown age. While this study did not analyze the mitochondrial genome, their success of recovering nuclear DNA confirmed my belief that there is a greater chance of isolating mtDNA from this substance.

A Danish team confirmed the presence of host cells within calculus' rigid matrix of crystalline calcium phosphates and silicate, which protect the cells from the elements that normally degrade DNA. The calculus used in this study was obtained from a middle/late Neolithic human skull (Preus et al. 2011).

The four Haplogroups most often found among American Indians are A, B, C, and D. These haplogroups are identified by mutations in the Hypervariable Region I of the D loop and corresponding mutations in the coding regions of the mtDNA genome (Lorenz and Smith 1996; Smith et al. 2005). The A, C, and D haplogroup mutations found in the coding regions of the organelles are revealed by the presence or absence of restriction enzyme cleavage sites. The Haplogroup B mutation is a nine base pair (bp) deletion the between the cytochrome oxidase II and lysine tRNA coding regions of the mtDNA genome (Lorenz and Smith 1996; Smith et al. 2005). Haplogroup A is defined as the presence of a *Hae III* restriction site at nucleotide pair (np) 663; Haplogroup C is defined by the absence of a *Hinc II* site at np 13,259 and the presence of an *Alu I* site at np 13,262; and Haplogroup D is defined as the absence of an *Alu I* site at np 5,176 (Lorenz and Smith 1996; Smith et al. 2005).

Lorenz and Smith (1996) describe the geographic distribution of the Haplogroups A, B, C, and D among 497 individuals of modern American Indian populations in North America. The California tribes represented in their study were the California Penutian and the Washo. Seventeen of the samples were California Penutian; among this population, 11.8 percent were Haplogroup A, 41.2 percent were Haplogroup B, 5.9 percent were Haplogroup C, and 41.2 percent were Haplogroup D. The Washo population of their study consisted of 28 individuals; of the 28 individuals, Haplogroup A was absent, 53.6 percent were Haplogroup B, 35.7 percent were Haplogroup C, and 10.7 percent were Haplogroup D.

METHODS

Sample selection for this study was based on the quantity of calculus per individual burial. Approximately half of the calculus from each burial was reserved for future analysis. Precautionary measures to prevent contamination of the samples were followed, as described by Kemp and Smith (2010). In addition, the polymerase chain reaction (PCR) amplifications of the extraction and FFPE products and the haplogroup regions were processed at different times to reduce the chance of cross contamination of the samples. The mtDNA of the individuals working in the Central Washington University NAGPRA analysis lab have been analyzed to determine if any individual was Haplogroup A, B, C, or D, to rule them out as sources of contamination. All students working in the Genetic lab routinely analyze their DNA, so their data are on file for comparison to rule them out as possible sources of contamination.

The five calculus samples were subjected to the extraction process designed for bone because of the density of calculus. The Geneclean Kit for Ancient DNA Manual Protocol was followed, including the recommended Proteinase K pre-incubation. However, the incubation time was extended from 12-15 hours to 70 hours to increase DNA yields. All other steps were processed according to the extraction protocol. Negative controls for the extraction process were used for the five samples. The following are the weights of the calculus samples used in this study: Burial 98, 45.8 mg; Burial 103, 57.4 mg; Burial 138, 26.1 mg; Burial 139, 49.7 mg; and Burial 216, 44.3 mg, resulting in a total weight of all samples of 223.6 mg. The combined weights were slightly less than the lowest starting weight recommended in the protocol for bone, 240-400 mg. Therefore, a master mix of the recommended solutions was prepared and

divided equally among the five samples for the Proteinase K pre-incubation. In an effort to increase the DNA yields of the samples, 10 microliters of each extraction product and negative controls were amplified with QIAGEN REPLI-g for Formalin Fixed and Paraffin Embedded tissues (FFPE). This product replicates smaller segments of the mtDNA genome (Joseph Lorenz, personal communication 2010).

The extraction products and the FFPE products for each of the five samples were amplified using human-specific primers for the four American Indian Haplogroups A, B, C, and D. The primers and restriction enzymes used in this study are listed in Table 1. The Qiagen Fast Cycling PCR Kit was used for all PCR amplifications. The total PCR volume of each sample was 20 microliters: 5 microliters o extraction product, 1 microliter each of forward primer and reverse primer, 10 microliters of master mix, and 3 microliters of sterile distilled water. The samples PCR products were amplified in a thermocycler. The amplification process consisted of three stages: stage 1 was 95° C for 10 minutes; stage 2 was 96°C for 30 seconds, 60° C for 30 seconds, and 68°C for 1 minute (for 40 cycles); and stage 3 was 72° C for 1 minute and 4° C for 2 minutes. Five microliters of the PCR products were electrophoresed on 6 percent acrylamide gels, then stained with ethidium bromide and viewed on a UV illuminator to verify the presence or absence of bands that signify the regions amplified.

Restriction digests were done on the samples that amplified for the desired regions of the mtDNA genome to determine the presence or absence of the restriction sites that identify Haplogroups A, C, and D. For the restriction digests reactions, 4 microliters of the PCR product was reserved to be run as an undigested controls. For the haplogroups that have only one restriction site, the reaction was set up with 10 microliters of PCR product, 3 microliters of respective buffer, 16 microliters of distilled water, and 1 microliter of the respective restriction digest, for a total volume of 30 microliters per reaction. For the haplogroup with two restriction sites, the PCR product was reduced to 5 microliters for each of the two digest reactions, and the distilled water was increased by 5 microliters with the same amounts of buffer and restriction enzyme as previously described, so that the final restriction digest solution was 30 microliters. The reactions were placed in a heating block set at 37° C for a minimum of 8 hours. The digested and undigested products were electrophoresed on 6 percent acrylamide gel, then stained with ethidium bromide to determine the presence or absence of the restriction sites. The samples that amplified for Haplogroup B region were electrophoresed alongside positive and negative controls. More specifically, two PCR products were used, one from an individual that is not Haplogroup B and one from an individual that is known to be Haplogroup B, to determine the presence or absence of the 9 bp deletion in the calculus samples.

To confirm the results of this study, I went back to Burials 139 and 216 extraction products and the FFPE product for Burial 98 and performed a second PCR of the D region, and then ran a restriction digest for the Alu I cleavage site.

RESULTS

Mitochondrial DNA was isolated and analyzed from calculus obtained from the skeletal remains of five individuals. Three of the five samples typed as Haplogroup D. The results of both the extraction and FFPE amplifications of the four regions that contain the identifying sequences of the haplogroups and the restriction digests are presented separately. It should be noted that none of the samples amplified for all four of the regions.

The result of the extraction samples amplified for the four haplogroup regions are listed in Table 2. Burial 98 amplified for Haplogroup B. Burial 139 amplified for Haplogroup D. Burial 216 amplified for Haplogroups B and D. The negative control for Burial 139 amplified for Haplogroup B.

The results of the amplification of the FFPE samples are listed in Table 3. Burial 98 amplified for Haplogroups B, C, and D. Burial 216 amplified for Haplogroup B. The negative control for Burial 98 amplified for Haplogroup C, and negative controls for Burials 138 and 139 amplified for Haplogroup D.

The Haplogroup D restriction digests of extraction products for Burials 139 and 216, as well the negative control for Burial 216, revealed the absence of the *Alu I* restriction site at np 5,176. This identifies the individuals from Burials 139 and 216 and the contaminate present in the Burial 216 negative control as Haplogroup D.

The Haplogroup D restriction digest of the FFPE product for Burial 98 and the negative controls for Burials 138 and 139 did not have a restriction cleavage site at np 5,176. Therefore, Burial 98 and the negative controls are Haplogroup D. The restriction digests of the second PCR of the Haplogroup D region used to check these findings confirms that Burials 98, 139, and 216 are Haplogroup D.

The results of the restriction digests for Haplogroup C included the FFPE PCR products for Burial 98 and its negative control. Both samples were missing the *Alu I* restriction cleavage site np 13,262, and the *Hinc II* restriction cleavage site at np 13,259 was present. Therefore the Burial 98 individual is not Haplogroup C; nor was the source of the contamination present in the negative control of Burial 98.

The samples that amplified for the Haplogroup B region from both the extraction and FFPE products were the same: Burials 98 and 216, and the negative control for Burial 139. None of the samples were positive for the 9 bp deletion. Therefore, the individuals from Burials 98 and 216 are not Haplogroup B. Nor is the contamination present in the negative control from a Haplogroup B individual.

None of the individuals who work in the NAGPRA analysis lab or those who work in the Genetic lab belong to Haplogroups A, B, C, or D. Therefore none of these individuals are a source of contamination to the calculus samples.

DISCUSSION

Three of the five samples used in this study typed as Haplogroup D, which is a haplogroup frequently found among modern populations of American Indians in the northern California area.

It was not possible to determine that there are not multiple haplotypes, because the Haplogroup A region did not amplify. However, those samples that amplified for more than one region did not result in multiple haplotypes per individual burial. Processing the extraction and FFPE products, as well the various haplogroup regions, on different days seemed to reduce the opportunity of cross-contamination.

There was intermittent cross-contamination among the samples that were processed at the same time. For example, the restriction digests for Haplogroup C of Burial 98 and its negative control from the FFPE products were both determined not be Haplogroup C.

There was not much of a difference between the extraction and FFPE products; both had four samples that amplified. While only the FFPE products amplified for Haplogroup C, in this instance there was not a great benefit to amplifying the entire mtDNA genome.

CONCLUSION

Calculus is a viable substance for mtDNA analysis of skeletal remains that range from the present to 1,000 years old. All three burials for which the mtDNA amplifications were successful have been assigned to Haplogroup D, which is a haplogroup that has a high frequency in the northern California area (Lorenz and Smith 1996). Burials 98, 139, and 216 did not display multiple haplogroups. There was intermittent contamination of the negative controls.

This study demonstrates benefits and limitations of using calculus as an alternate source for DNA analysis of skeletal remains. First, removing calculus from the teeth of skeletal remains is less destructive to the individual remains than the traditional method of DNA analysis. This feature may encourage some tribes to allow DNA analysis. Second, the sample size needed for analysis is much smaller than the quantity traditionally used for analysis. Third, calculus offers an indirect method of DNA analysis that is more closely linked to a specific individual than other sources used for indirect DNA analysis.

The following are the limitations of using calculus as a source for DNA. First, not all tribes will be willing to subject the remains of possible ancestors to this type of analysis. Second, the human remains may be compromised when removing the calculus. Skeletal remains can be fragile for various reasons; e.g., remains are desiccated, or enamel is crazed. Third, all possible haplogroups may not amplify from one source, which could result in an inability to assign remains to a haplogroup.

The next step in future research of calculus as a source for mtDNA could be studies that use larger sample sizes, blind testing of samples with known mtDNA haplotypes, and samples of varying ages to verify the efficacy of calculus as alternative source for mtDNA analysis.

REFERENCES CITED

Eggen, K. H., and G. Rölla,

1985 The Purification of a Protein Component in Extracts from Supragingival Dental Calculus. *Scandinavian Journal of Dental Research* 93(5):426–431.

Jenkins, Dennis L.

2007 Distribution and Dating of Cultural and Paleontological Remains at the Paisley Five Mile Point Caves in the Northern Great Basin. In *Paleoindian or Paleoarchaic? Great Basin Human Ecology at the Pleistocene/Holocene Transition,* edited by Kelly E. Graf and Dave N. Schmitt, pp. 57–81. University of Utah Press, Salt Lake City.

Kawano, S., Toshiaki Tsukamoto, Hiroko Ohtaguro, Hirofumi Tsutsumi, Tokyo Takahashi, Ichiro Miura, Rei Mukoyama, Hirofumi Aboshi, and Toshinobu Komuro

1995 *Sex Determination from Dental Calculus by Polymerase Chain Reaction (PCR)*. Department of Legal Medicine, Nihon University of dentistry, Tokyo, Japan.

Kemp, Brian M., and David Glenn Smith

2010 Ancient DNA Methodology: Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota." *Human Biology: The International Journal of Population Genetics and Anthropology* 82:227–238.

Kerr-Siefkin, Susan

1993 Dental Trauma in Prehistoric Hunter-Gatherers of California: A Comparison of Human Populations with Terrestrial and Marine-Oriented Subsistence Patterns. Unpublished Master's thesis, Department of Anthropology, University of California Santa Barbara.

Lorenz, Joseph G. and David Glenn Smith

1996 Distribution of Four Founding mtDNA Haplogroups among Native North Americans. *American Journal of Physical Anthropology* 101:307–323.

Marsh, P. D. and D. J. Bradshaw

1995 Dental Plaque as a Biofilm. *Journal of Industrial Microbiology* 15:169–175.

Mergenhagen, Steven. E., Ann L. Sanberg, Bruce M. Chassy, Michael J. Brennan, Maria K. Yeung, Jacob A. Dokersloot, and John O. Cisar

1987 Molecular Basis of Bacterial Adhesion in the Oral Cavity. *Reviews of Infectious Disease* 9:S467-S474.

O'Rourke, D. H., M. G. Hayes, and S. W Carlyle

2000 Ancient DNA Studies in Physical Anthropology. *Annual Review of Anthropology* 29:217–242.

Preus, Hans R., Ole J. Marvik, Knut A. Selvig, and Pia Bennike

2011 Ancient Bacterial DNA (aDNA) in Dental Calculus from Archaeological Human Remains. *Journal of Archaeological Science* 38:1827-1831.

Smith, David Glenn, Ripan S. Malhi, Jason Eshleman, Frederika A. Kaestle, and Brian M. Kemp
2005 Mitochondrial DNA Haplogroups of Paleoamericans in North America. In *Paleoamerican Origins: Beyond Clovis*, edited by Robson Bonnichsen, Bradley T. Lepper, Dennis Stanford, and Michael R. Waters, pp. 243–254. Center for the Study of the First Americans, College Station, Texas.

Teymoortash, Afshin, Ana C. Wollstein, Burard M. Lippert, Roswitha Peldszus, and Jochen A.Werner
2002 Bacteria and Pathogenesis of Human Salivary Calculus. *Acta otolaryngologica* 122:210–214.

Table 1. Primers and restriction enzymes used in study.

HAPLOGROUP	PRIMER	SEQUENCE 5' → 3'	RESTRICTION ENZYME	RESTRICTION SITE
A	H663 L663	ACATCACCCCATAAACA GTCCTTTTGATCGTG	+ Hae III	663
B	H8215 L8297	ACAGTTTTCATGCCCATCGTC ATGCTAAGTTAGCTTTACAG		9 bp deletion
C	H13393 L13232	TCCTATTTTCGAATATCTTGTTCC CGCCCTTACACAAAATGACATCCAA	+ Alu – Hinc II	13,262 13,259
D	H5230 L5120	AAAGCCGGTTAGCGGGGGCA TAACTACTACCGCATTCCTA	– Alu I	5,176

Table 2. Results of the PCR Amplifications from Extractions.

BURIAL	HAP A	HAP B	HAP C	HAP D
98		Positive		
103				
138				
139				Positive
216		Positive		Positive
98 NEG				
103 NEG				
138 NEG				
139 NEG		Positive		
216 NEG				Positive

Table 3. Results of the PCR Amplifications from FFPE Amplifications.

BURIAL	HAP A	HAP B	HAP C	HAP D
98		Positive	Positive	Positive
103				
138				
139				
216		Positive		
98 NEG			Positive	
103 NEG				
138 NEG				Positive
139 NEG		Positive		Positive
216 NEG				