Amplification of Ancient Nuclear DNA From Teeth and Soft Tissues

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The ability to isolate and sequence gene fragments from remains associated with archaeological sites is a valuable tool for the reconstruction of a population's history. Because of the extreme sensitivity of the PCR and the very small numbers of intact DNA templates in ancient DNA extracts it is important to insure that ancient samples are not contaminated by contemporary DNA. Teeth form a natural barrier to exogenous DNA contamination and the DNA recovered from teeth seem to be lacking in most of the inhibitors to enzymatic amplification of ancient DNA. We have demonstrated that teeth are the tissue of choice in the evaluation of DNA sequences from 18 Egyptian mummies.

The opportunity to study ancient populations at the gene level has been made possible by the recent ability to amplify fragments of DNA from archaeological specimens.[1–7] The ability to determine ancient DNA sequences from these fragments allows us to address a number of important questions regarding a population's history. Some of these include the number and direction of migrations, assimilation of or replacement by invading groups, and specific gene frequency changes of either disease-associated alleles or arbitrary loci. These changes may be monitored in a population at many points over substantial time periods, possibly long enough to identify meaningful rates of nucleotide change.

In a few areas of the world where the climate and related factors have been conducive to preservation (e.g., Egypt, Peru, northern Chile, the southwest United States, the peat bogs of Europe, and Florida) it is possible to recover ancient human soft tissue remains.[8] However, in these areas and in others throughout the world it is more likely that the only remaining tissue associated with an archaeological site will be bones or teeth. The ability to use bone and teeth as sources of amplifiable DNA from human remains has been previously reported.[2,5,6,9,10] Using a simple procedure we have compared the ease of extraction, the resulting quality of the DNA, and ultimate reliability of data obtained from soft tissue and teeth of ancient Egyptian mummies. We have demonstrated that teeth provide the tissue of choice for recovering biologically active ancient DNA free of contaminating contemporary DNA and PCR inhibitors.

MATERIALS AND METHODS

Sample collection from mummies

During the excavation of the bodies, care was taken to avoid any inadvertent contamination of the ancient material with contemporary sources of DNA (e.g., hair, skin flakes, etc.) by covering the head, arms, and hands of the excavator. As the wrapped bodies were removed from the ground they were transported to the examination and study area by individuals covered with hats, and wearing long sleeves and gloves. The bodies were unwrapped by one of the excavation team members who was gloved and masked. Samples were collected using sterile disposable blades, forceps, and collection tubes. Multiple small samples typically consisting of soft tissues from various areas of the body such as scalp hair, muscle, and internal tissues were collected from each mummy in addition to bone and teeth. These samples were sealed in either 15-ml conical centrifuge tubes or 1.5-ml Eppendorf tubes. The group of tubes representing samples from a single body was sealed in a plastic bag for transport to the laboratory. At the laboratory, bags representing the 20 individual mummies included in this study were opened one at a time in a clean laminar flow hood by a gloved and masked individual. Small aliquots of soft tissue were prepared directed into tubes for DNA extraction procedures. A single tooth from each individual was transferred to a new sterile tube for use in the DNA extraction process outlined below.

DNA Extraction from Soft Tissue

A very small piece of tissue (~1.0-mm square) was placed in a Eppendorf tube in 500 μl of a 5% chelex[11] solution (wt/vol in H_2O) and crushed with a pipet tip. This sample was vortexed for 1 min, incubated at 56°C for 15 min, vortexed for 1 min, brought to 95°C for 10 min, vortexed for 1 min, and then centrifuged at 12,000*g* for 3 min. A portion of the supernatant (typically 15 μl) is used in the PCR reaction.

DNA Extraction from Teeth

The outside of the tooth is cleaned with filtered compressed air and a small amount of the external material is removed using a small drill and bit. A small bore hole is made into the sample,

3:244–247©1994 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/94 $5.00

usually in the region between the crown and the root to expose the pulp area of the tooth. All of the powder resulting from the cleaning of the outside surface of the tooth is removed and disposed. A clean drill bit of slightly smaller diameter is placed on the drill, cleaned by low-speed rotation in three serial rinses in sterile, distilled H_2O, rinsed in 95% alcohol, and then flamed and cooled on dry ice. The drill bit is reintroduced at high speed (30,000 rpm) into the previously drilled area. A small portion of the newly powered material is collected using a wetted pipet tip and processed in Chelex as described above.

Amplification of Nuclear Genes

PCR amplification was performed in a volume of 25 μl on a Perkin-Elmer Cetus 480 Thermal Cycler. Forty cycles of a basic temperature profile consisting of a denaturation step at 94°C for 30 sec, an annealing step at 52°C for 1 min 30 sec, and elongation at 72°C for 30 sec was performed. The reaction mixture contained 67 mM Tris (pH 8.8), 2 mM $MgCl_2$, 16.7 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 0.2 mM dNTPs, 1 μM each primer and DNA (usually 15 μl of the Chelex extraction supernatant). The primers used are from the DQ A1 gene of the HLA region on chromosome 6 and have the sequence 5′ GTG CTG CAG GTG TAA ACT TGT ACC AG 3′ and 5′ CAC GGA TCC GGT AGC AGC GGT AGA GTT G 3′.[12] The expected fragment size is between 239 bp and 242 bp. Control reactions were set up as follows. Negative controls: In place of the ancient DNA, an equivalent volume of: (1) a blank Chelex solution supernatant (subjected to the same protocol as the ancient DNA extractions); (2) Chelex solution supernatant in which the drill bit had been rinsed after cleaning between samples (to insure there has not been any carryover from sample to sample); (3) water only. Positive controls: (1) contemporary DNA in water; (2) contemporary DNA in the presence of a blank Chelex supernatant. Separate micropipetters were used in the DNA extractions, preparation of the PCR reactions, and PCR product evaluations. The PCR setup, amplification, and evaluation are each done in a separate room. Evaluation of the PCR product was performed on 2.5% NuSieve agarose in Tris-acetate buffer containing ethidium bromide. Positive bands for secondary amplifications were cored with a Pasteur pipet, placed in a 0.5-ml Eppendorf tube, diluted to 100 μl in distilled water, and heated to 85°C for 10 min.

Test for Inhibition

Samples were tested for the presence of an inhibitory substance by attempting amplification of contemporary DNA in the presence of the Chelex extracts of the ancient DNA. Typically, 9.8 μl of the ancient Chelex extraction supernatant was combined with 5μl of contemporary DNA (total equals 10 ng contemporary DNA in distilled H_2O) and amplified as outlined above. For reactions where inhibition was present, the original extraction supernatant was diluted 1 : 10 into a fresh 5% Chelex solution, heated to 94°C for 5 min, vortexed, and centrifuged. Occasionally, a second round of dilution (final 1 : 100) was necessary to produce a positive amplification as evidence by the presence of "primer dimer" and/or the specific product.

Cloning and Sequencing

Plasmid clones of both primary and secondary amplification products were prepared using the pCR II TA cloning vector (Invitrogen, Inc., San Diego, CA) according to the manufacturers protocol. Plasmids with potential inserts were identified as white colonies and were further evaluated for the presence of inserts by plasmid minipreps[13] and gel electrophoresis. Clones shown to be unique by SSCP analysis were sequenced by standard double-stranded dideoxy methods.[14]

RESULTS AND DISCUSSION

During the 1992 field season of the Brigham Young University Seila Egypt expedition, soft tissue, bone, and teeth samples were collected at the moment of the discovery from 142 Egyptian mummies. these were located in a cemetery at Fag el Gamous near the village of Seila in the Fayum. This Greco-Roman period (~200 BC–800 AD) cemetery is large, covering ~300 acres and contains at least 150,000 bodies.[15] Brigham Young University has excavated at this site and at the nearby pyramid of Sneferu since 1981.[16] The term mummy is used interchangeably with burial and body in this report, although not all burials were prepared for interment by technical mummification procedures. Most of the mummies recovered from this cemetery appear to be naturally desiccated by the sand, although some show evidence of having been prepared with salts prior to burial. The bodies are usually found wrapped with numerous layers of burial cloth.

DNA was isolated from both teeth and soft tissue from the mummies. The DNA from the teeth extractions generally gave positive amplifications without further modifications. Of the 20 DNA samples extracted from teeth, 18 showed amplification of appropriate size fragments using primers to the HLA DQ A1 region (Fig. 1). None of the soft tissue extractions produced amplified fragments on the initial attempt. These tissue samples often contained a PCR inhibitor[1] as evidenced by no amplification product in the primary reaction, lack of primer dimer, and inhibition of amplification of contemporary DNA in the presence of ancient DNA extracts. As samples were diluted, the primer dimers reappeared and amplification of contemporary DNA was no longer inhibited (Fig. 2). After dilutions of 1 : 10 or 1: :100 in fresh extraction medium some of the soft tissue samples produced amplified fragments. If there was any visible color in the extraction supernatant, usually light to dark yellow, the amplification was inhibited.

Secondary amplifications were analyzed by single-strand conformational polymorphism (SSCP)[17,18] to estimate the distribution of alleles in the sample population. In the 18 samples derived from teeth, 12 variant alleles were iden-

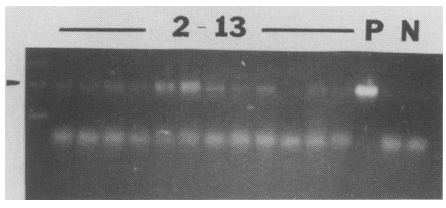

FIGURE 1 NuSieve agarose gel (2.5%) showing the amplification of an ~243-bp fragment of the HLA DQ A1 locus from 12 ancient Egyptian mummies. (Lane *1*) 123-bp marker. (Lanes *2–13*) Ancient DNA samples. Notice that the amplification in lane *11* was not successful. (Lane *P*) Contemporary DNA control. (Lane *N*) Negative control, Chelex extraction buffer, and negative control, distilled H_2O.

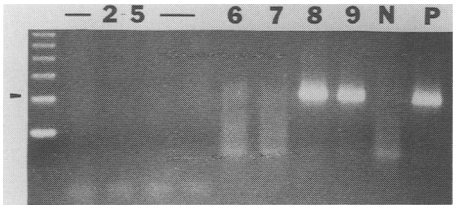

FIGURE 2 NuSieve agarose gel (2.5%) demonstrating the inhibition of amplification of DNA from soft tissue. (Lane *1*) 123-bp marker. (Lanes *2–5*) Undiluted extract from tissue including 10 ng of contemporary DNA. (Lanes *6,7*) A 1 : 100 dilution of a tissue extract with 10 ng of contemporary DNA. (Lanes *8,9*) A 1:100 dilution of a tissue extract with 10 ng of contemporary DNA. (Lane *N*) Negative control, distilled H_2O. (Lane *P*) Positive control, 10 ng contemporary DNA in Chelex buffer.

tified by their altered mobility in the SSCP gel. Possible contamination by contemporary DNA corresponding to any of the individuals involved in the excavation or the laboratory procedures was monitored in this way. Contemporary DNA from each of these individuals demonstrated patterns of SSCP gels different from any of the ancient samples examined.

Eighteen subclones representing three ancient individuals from different alleles demonstrated by SSCP were sequenced. HLA types[19] were determined from the sequence information (in prep.). The positive control DNA used throughout this study is known to be homozygous type 1.2. Allele types 1.3 and 4 were identified in subclones from the PCR products of the three ancient DNA samples. One individual was a 1.3/4 heterozygote, the other two were homozygous for allele 4. Three of the clones sequenced that most closely corresponded to allele type 4 had different single base substitutions, probably caused by error during PCR because of damaged templates. These would normally be averaged out during direct sequencing. However, because of the difficulty of determining phase in heterozygotes, cloning and then sequencing was performed.

A number of technical problems have made it difficult to obtain large numbers of reliable amplification products from human remains. Possible contamination of the ancient sample with contemporary DNA is a major obstacle. This is particularly troublesome when studying ancient human DNA rather than other animal or plant DNA, because the most probable source of contaminating contemporary DNA that would interfere with subsequent analysis would also be of human origin. It is most likely that if the ancient DNA is contaminated with contemporary DNA, it would occur during the collection process and subsequent handling, or during the laboratory manipulations.[3,9] Analysis of the resulting amplification product(s), including sequencing, may not allow differentiation of contemporary from ancient human DNA.

From the outset of this study extreme effort was made to recover ancient biological samples free from the contamination by contemporary DNA. In the field, precautions included covering the investigators skin and hair during the process of excavation and collection of samples. The mummies were usually handled by a single investigator, with four persons being the maximum number of individuals in contact with the mummy during the entire process of excavation, sample collection, and laboratory evaluations. Samples were collected using sterile disposable instruments and collection vials. In the laboratory these samples were aliquoted by the same individuals who collected them in the field. This was done in a carefully controlled environment including laminar flow hoods. Precautions at the laboratory bench included use of separate pipetters for pre-PCR and post-PCR manipulations, ultraviolet radiation of all reagents, and the use of separate rooms for the initial setup, amplification, and the analysis of the amplification products by gel electrophoresis, subcloning, and sequencing. Multiple negative controls were included during the amplification to monitor for external contamination of contemporary DNA.

The use of teeth as the preferred source of biologically active ancient DNA for use in the PCR is demonstrated in this report. Being able to physically remove any contaminating DNA from the surface of the tooth and then drilling into an area that has been cleaned helps ensure the integrity of the ancient DNA. In addition to being able to eliminate contemporary DNA from the outside of the tooth it seems that the resulting DNA is much more susceptible to amplification by PCR. It has been observed that most ancient DNA samples from soft tissues used to date have some type of inhibitory substance that co-purifies with the DNA, possible oxidative products of sugar.[1] It has been shown that a high amount of oxidative damage can be present in ancient DNA isolated from soft tissue, resulting in altered or missing bases, damaged sugar residues, and intermolecular crosslinks.[1,20] Various methods have been used to separate the inhibitor from the DNA or to overpower the inhibitor in the reaction.[1] Our results suggest that the DNA in ancient teeth may be protected from some of the inhibitors that are present in ancient soft tissues.

Boiling in Chelex was the primary method of DNA extraction for use in the PCR reaction. Compared with standard DNA extraction procedures, this protocol contains fewer steps. The probability of contamination from outside sources of DNA is decreased by the limited handling and a reduction in the number of different reagents that come in contact with the ancient sample. The ability to recover biological active DNA from ancient DNA has been enhanced over other procedures by using the Chelex extraction method.

It has been shown that in certain instances when using HLA DQ A1 primers for PCR, including starting with a very small amount of DNA, which is relevant to this study, that "allele dropout" may occur.[21] This effect can be minimized by optimizing the reaction conditions, particularly the denaturation temperature of the cycling reaction. Some HLA DQ A1 alleles have a higher CG content than others, and it is therefore important that the denaturation temperature reaches 94°C. Also, stochastic fluctuations when dealing with a small number of intact starting templates may allow only one allele to be amplified in the reaction mixture. An attempt was made to reduce this problem by performing multiple reactions from each ancient DNA sample. Most of the ancient samples in this study demonstrated two alleles.

It is interesting that of the alleles sequenced, either directly or following cloning, there was a lack of scrambled or derived sequences as a result of "jumping PCR" that has been observed in other studies of ancient DNA.[3,20] Perhaps the ancient DNA obtained from teeth lacking polymerase inhibitors is a more reliable template for subsequent PCR manipulations. The ability to isolate

noncontaminated and biologically active DNA from ancient teeth which is demonstrated by this study now makes it possible to address many important questions regarding the study of ancient population genetics.

ACKNOWLEDGMENTS

Part of this work was supported by grants from the Marriott Corporation and Brigham Young University for the excavation at Fag et Gamous and the Molecular Biology Working Group. Dr. Marek J. Kaliszewski, deceased, participated in the excavation and preliminary laboratory work. We thank the Egyptian Antiquities Organization (EAO) for their help and cooperation, particularly Dr. Mohammed Bakr, Direction of EAO; Mr. Mutawe Balbousch, Director of Middle Egypt; and Mr. Ali Bazidi, director of the Fayum. We also thank Mr. Atef William, the EAO inspector who assisted the team in this work.

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Received August 30, 1993; accepted in revised form December 16, 1993.