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Creating a Model for Ancient DNA Isolation from Contaminated Archaeological Materials Using Sodium Hypochlorite Pretreatment and Enzymatic Predigestion.

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Abstract: The retrieval of DNA from archaeological or forensic skeletal remains provides valuable data for analysis, but it faces specific challenges. The study aims to develop a standardized method for isolating ancient DNA, with applications in archaeology, genetic ages, disasters, and human remains. One crucial challenge is the presence of contemporary contamination on bone and teeth surfaces, which can lead to misleading outcomes and false positives if not addressed before DNA extraction. To mitigate this contamination, Ancient DNA (aDNA) researchers and forensic scientists have utilized various techniques, including using bleach (sodium hypochlorite - NaOCl) to "eliminate" the contaminating DNA. However, caution is advised, as this treatment has the potential to damage a significant portion of the sample's native molecules, as indicated by some earlier research.To further investigate this subject, the study employed samples of both human and animal remains and subjected them to sodium hypochlorite treatment to eliminate contamination. The findings align with prior research, showing a notable decrease in human contamination after applying the bleach pretreatment. However, it's essential to note that this reduction in contaminant DNA comes with a trade-off, resulting in a decrease in the complexity of endogenous DNA.

Keywords: Ancient DNA, DNA contamination, pretreatment, sodium hypochlorite, DNA extraction

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Introduction

The discovery of DNA that can endure for extended periods, spanning hundreds of thousands of years, within skeletal remains (1, 2), and sedimentary deposits has significantly expedited the use of ancient DNA analysis in evolutionary research. Nevertheless, isolating small amounts of extensively damaged DNA from such substances is complex. This is due to the challenge of effectively separating short DNA fragments from various organic compounds, such as humic acids. These compounds hinder the enzymatic DNA modifications necessary before sequencing (3, 4). Bones and teeth discovered at archaeological sites often contain fragments of ancient DNA, which allows researchers to directly study the genetic makeup of organisms that lived tens to hundreds of thousands of years ago. However, the DNA preservation in these remains varies significantly and depends on their age and the environmental conditions at the

excavation site (5, 6). Scientists believe DNA preservation is related to the interaction of nucleic acids with two key components: hydroxyapatite, found in the inorganic fraction of bones, and collagen, located in the organic fraction of bones and teeth (5, 6, 7). DNA attaching to hydroxyapatite is facilitated by electrostatic interactions between calcium ions, which carry a positive charge, and the phosphate groups of the DNA backbone, which possess a negative control. The rate of depurination, a chemical reaction that can cause damage to DNA, is seen to be slower in DNA that is bound to hydroxyapatite compared to unbound DNA (8). Additionally, hydroxyapatite can bind and deactivate nucleases (enzymes that break down DNA) (9). On the other hand, the mechanism by which DNA interacts with collagen and its significance in the enduring conservation of DNA within ancient tissue needs to be more adequately comprehended. Nevertheless, DNA-collagen complexes, which form spontaneously in aqueous solutions in laboratory settings, could play a role in safeguarding DNA within ancient specimens (7, 8, 9, 10). The analysis of ancient DNA encounters challenges arising from the intricate interplay between DNA and the bone/tooth matrix. This interplay encompasses not only endogenous DNA, which refers to DNA present in the organism before or at the time of death, but also exogenous DNA, including that originating from microorganisms that infiltrate the bone/tooth matrix during decomposition. Except for a few cases (11, 12, 13), most molecules in ancient DNA extracts typically comprise microbial DNA, accounting for over 95% of the total. We delved into creating extraction methods incorporating particular enzymes to conquer this obstacle. The primary objective was to devise a novel approach for isolating ancient DNA, enabling its use in archaeological genetic studies, disaster analyses, and human remains investigations.

Materials and methods

Bone and teeth samples collection

In this research endeavor, a total of four samples were utilized—two from an adult male skeleton (femur bone and tooth) and two from a child skeleton (femur bone and tooth). These specimens were sourced from the Lagash cemetery in Dhi Qar Governorate, as depicted in (Figure 1) and date back over a millennium.(The age was determined by the American Archaeological Mission from the University of Pennsylvania, which excavated the site according to the archaeological methods approved for identifying antiquities). Moreover, animal samples were also included in the study. These encompassed a cow's femur bone, a corresponding tooth, and a set of bones from an unidentified animal skeleton. These animal samples, found in an exposed environment, have been preserved for several years. Additionally, an examination was conducted on portions of a cat's skull bones discovered in an outdoor setting. as shown in (Figure 2).

Figure (1): Variety of samples, which included a femur bone and teeth from an adult male skeleton, and a femur bone and a tooth from a child skeleton. These specimens were sourced from Lagash cemetery in Dhi Qar Governorate.

Figure (2): Part of a cat's skull bones was discovered in an open setting

Pre-treatment of samples

Every sample underwent thorough treatment with a 12% bleach solution for 10 seconds. The entirety of the bone surfaces underwent thorough sanding to eliminate any possible presence of foreign DNA. Subsequently, a series of three rinses with distilled water was conducted. A final sonication wash was conducted employing 100% ethanol, and after that, the bone specimen was positioned within a sterile fume hood to undergo overnight air drying. Subsequently, implement the utilization of Deep Freeze. The materials were evenly partitioned and pulverized on the subsequent day utilizing two distinct methodologies.

a- Utilizing a dental drill at a low speed to prevent sample heating.

b- Utilizing a specialized device designed for grinding bones and teeth. It is imperative to prepare all dilutions under strictly aseptic conditions. As shown in (Figure 3).

Figure (3): The devices used for grinding teeth and bone samples

DNA extraction

The procedures for extracting ancient DNA should aim to enhance DNA retrieval while reducing the influence of PCR inhibitors. It's advisable to streamline the steps involved in the process to decrease the potential for contamination. 1g of bone or tooth powder was added into a 1.5 ml tube, and then 1 ml of bleach solution (crafted in the lab using 0.5% sodium hypochlorite in water, suitable for a one-month timeframe) was introduced. Following this, either vortexing or shaking was performed, followed by a 15-minute rotation period. The mixture was centrifuged for 3 minutes, the liquid portion was disposed of, and the sediment was gathered. To the residue, 1 ml of distilled water was introduced, gently vortexed, and rotated for another 3 minutes. It was then centrifuged at the highest speed for 3 minutes, the supernatant was discarded, and the sediment was retrieved. This sequence was repeated thrice. To the sediment, 1 ml of predigestion buffer (created in the lab with 0.45M EDTA, pH 8.0, and 0.25 mg/ml proteinase K at pH 8) was added, vortexed, and then the sample was subjected to incubation at 50 °C for 15-30 minutes. The ensuing steps were proceeded with: centrifuging for 3 minutes at the highest speed, dispose of the supernatant, and gather the sediment (14). The sediment was taken and placed on it 500ml stain buffer (10 mM Tris, pH 8; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS) and 20 mg/mL Proteinase K at 56 8°C with gentle agitation (overnight). The next day, the mixture of 500ml phenol/chloroform/isoamyl alcohol (25:24:1) was added. The supernatant was then transferred into a 1.5ml tube and washed with 1ml of absolute ethanol. The sample tube was placed in a -20°C environment for 30 minutes and subsequently subjected to centrifugation at 10000-15000 for 12 minutes, after which the supernatant was discarded. Following this, 1ml of 70% ethanol was added and mixed by vortex for 10 seconds. The sample tube was placed in a centrifuge at 10000-15000 for 5 minutes, and the supernatant was discarded. The sample was allowed to dry on filter paper, and 25 µTE buffer was added, mixed by vortex for 10 seconds. Finally, the sample was placed in a 4°C environment for 2 hours.

Gel electrophoresis

The gel contained 1% agarose and Diamond Nucleic acid Dye visualized DNA. We weigh 0.6 grams of agarose powder and dissolve it in 60 milliliters of TBE buffer. We then place it in the microwave for 1 minute. After

it cools down, we add the dye and pour it into the gel electrophoresis tank, making sure to place the comb. We wait until the gel solidifies and then load the sample along with the loading dye. We connect the electrophoresis apparatus, turn it on, and set it to 70 volts for 40 minutes. After that, we read the results using UV light.

NanoDrop Spectrophotometer

DNA analysis and quantification have become routine procedures in molecular biology laboratories as a foundational step for various experimental protocols. Measuring sample absorbance at 260 nm is a frequently employed technique for estimating nucleic acid concentration(15). The absorbance ratios of 260/280, 260/230, and 260/325 are commonly used in assessing DNA purity and identifying potential contaminants in biological samples during DNA extraction(16). The concentration and purity were measured using the NanoDrop device.

Validation of the nanoDrop DNA quantification method

The evaluation parameters for DNA measurements in samples' micro volumes (1 µL) encompassed several aspects. These included the working range (linearity) of the measurements, the detection and quantification limit determined using a blank sample treated with DEPC, the precision of the measures under conditions of repeatability and reproducibility, the trueness of the measurements assessed through bias and recovery percentage, and the measurement stability (17).

Results and discussion

We experimented on four samples, including a femur bone and a tooth from an adult male skeleton, as well as a femur bone and a tooth from a child skeleton. These specimens were sourced from Lagash cemetery in Dhi Qar Governorate, dating back over a thousand years. Additionally, we examined animal samples comprising a cow's femur bone, a tooth from the same animal, and a group of bones from an animal skeleton of an unknown type, dating back several years and found in an exposed environment. Furthermore, we analyzed part of a cat's skull bones discovered in an open setting.

Prepare the samples for analysis, as stated in the materials and method involving cleaning, grinding, and extracting the bones. During the extraction process, we initially used bleach treatments, which successfully yielded high concentrations of bone and teeth from the animal samples. However, when it came to the ancient human samples, the bleach treatment had an adverse effect, resulting in deficient concentrations and yielding negative results. This outcome could be attributed to sample deterioration, storage conditions, or the impact of bleach on ancient human specimens. To further explore this issue, we conducted a similar experiment on the human sample without subjecting it to bleach treatment, but unfortunately, the result remained negative, as shown in (Figure 5) and (Table 1).

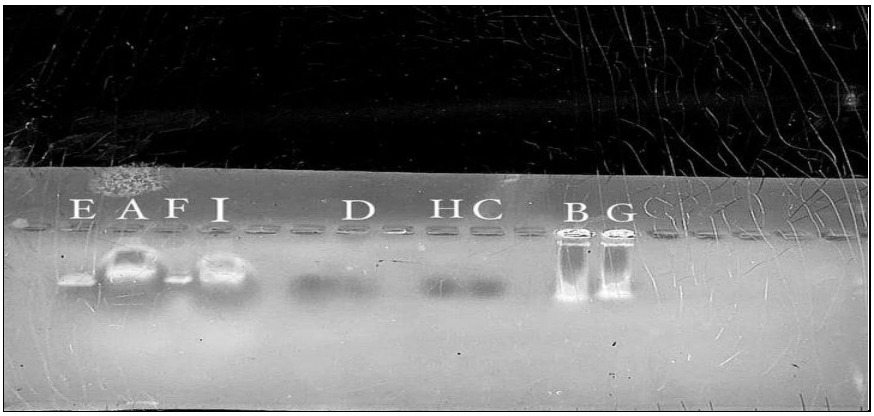

Figure (5): Agarose gel electrophoresis of DNA extracted from the remains of ancient bone and teeth. The gel contained 1% agarose and DNA was visualized by Diamond Nucleic acid Dye. Letters refer to the samples analyzed (see Materials and Methods). Migration positions of molecular size markers are indicated in bp

Table (1): The effect of bleach treatment on the concentrations of different old samples of teeth and bones

SAMPLES	Concentration
A (teeth animal)	28.1ng/ µL
B (bone animal)	148.4ng/ µL
C (ancient bone)	0.00ng/ µL
D (ancient teeth human)	0.5ng/ µL
E (ancient teeth animal)	14.8ng/ µL
F (bone animal)	11.8ng/ µL
G (teeth animal)	83.6ng/ µL
H (ancient bone human)	3.1ng/ µL
I (ancient bone animal)	78.9ng/ µL

The preferred option for DNA analysis is usually biological material with exceptional preservation characteristics. This preference primarily stems from the solid protein-mineral framework found in bones, which effectively guards against environmental decay and biological breakdown (18). Teeth, in particular, stand out due to their durable composition, rendering them highly resistant to decay and temperature fluctuations. Notably, even under elevated temperatures and extended durations, DNA can be successfully extracted from teeth. Conversely, attempts to extract DNA from soft tissues often prove unsuccessful, especially when the body has experienced decomposition or incineration. The teeth' tough enamel and dentin covering act as a barrier, safeguarding the inner pulp from air currents and potential contaminants, thereby aiding DNA extraction (19). The most reliable approach to prevent sample contamination is strictly adhering to aDNA sterility protocols from the excavation stage onward. Contamination during collection poses a significant challenge, particularly for bones and teeth (20, 21). Even samples gathered using meticulous sterile procedures might still carry surface impurities from the environment in which they were deposited. Numerous methods have been proposed to eliminate surface contaminants from ancient bones and teeth. These methods encompass physically removing the outer layer, subjecting the surface to procedures such as washing or extended exposure to substances like water, EDTA, bleach, ethanol, acid, or

hydrogen peroxide, subjecting the sample to UV irradiation, and extracting the inner material (22). When the sample is well-preserved, bleaching bone powder seems to selectively degrade contaminant DNA more than endogenous DNA, as detailed in the materials and methods section.The results of our study revealed contrasting outcomes in the extraction process of skeletal remains from ancient human and animal samples. We observed the successful extraction of high concentrations of bones and teeth from the animal samples following the bleach treatment. However, the bleach treatment had a detrimental effect on the ancient human samples, leading to significantly lower concentrations(As shown in Table 1, samples C, D, H,) and the absence of a band in agarose gel electrophoresis (Figure 5). While in animal samples (A, B, I, G, E and F) the con. were high and the appearance of DNA in the agarose gel.

These findings align with previous studies investigating the challenges associated with extracting and analyzing ancient human skeletal remains. Several factors may contribute to the negative impact of bleach treatment on old human samples. Sample degradation is one possible explanation for the observed low concentrations and negative results. Over time, ancient human skeletal remains undergo degradation processes, including chemical changes and mineral loss, which can impair the efficacy of the extraction methods (Higgins and Austin, 2013)(23). Additionally, the preservation and storage conditions of the ancient human samples could play a crucial role in their susceptibility to bleach treatment. Variations in burial conditions, environmental factors, and handling practices throughout history may influence the degree of sample preservation.

The impact of bleach treatment on ancient human skeletal remains has been investigated in previous research. For example, a study by Watt *et al.*, (24). Decontamination techniques in old DNA analysis (24). To gain further insights, we conducted an additional experiment on the human sample without subjecting it to bleach treatment. Surprisingly, the result was also negative, suggesting that this ancient human sample may require alternative extraction methods.

Conclusion

In conclusion, our results demonstrate the challenges associated with the extraction process of ancient human skeletal remains compared to animal samples. Factors such as sample degradation, storage conditions, and the impact of acid treatment likely contribute to the observed low concentrations and negative results. These findings are consistent with previous studies highlighting the complexities of working with ancient human remains. Further research is needed to explore alternative extraction methods to overcome these challenges and improve the analysis of ancient human skeletal remains.

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