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Forensic Science International 154 (2005) 53–61
Forensic Science International
www.elsevier.com/locate/forsciint

Short communication

Use of bleach to eliminate contaminating DNA from the surface of bones and teeth

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Received 27 September 2004; received in revised form 24 November 2004; accepted 26 November 2004
Available online 2 February 2005

Abstract

The extraction of DNA from archaeological or forensic skeletal remains can provide quite powerful data for analysis, but is plagued by a unique set of methodological problems. One of the most important methodological problems to overcome in such analyses is the presence of modern contamination on the surfaces of bones and teeth, which can lead to false positives and erroneous results unless it is removed before DNA extraction is initiated. Ancient DNA (aDNA) researchers and forensic scientists have employed a number of techniques to minimize such contamination. One such technique is the use of bleach (sodium hypochlorite—NaOCl) to ''destroy'' contaminating DNA. However, a consensus on the optimum concentration of sodium hypochlorite to be used and the amount of time the bone or tooth should be exposed to it has not emerged. The present study systematically approaches the issue by introducing contamination to ancient bones (from ~500 BP) and determining which of several sodium hypochlorite treatments best eliminates surface contamination. The elimination of surface contamination from bone requires immersion in at least 3.0% (w/v) sodium hypochlorite (approximately equal parts of commercial bleach and water) for at least 15 min. Endogenous DNA proved to be quite stable to even extreme sodium hypochlorite treatments (6% for 21 h), suggesting that DNA adsorbs to hydroxyapatite in the bone and that this process facilitates the preservation of DNA in ancient skeletal remains.
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Keywords: Skeletal remains; Ancient DNA; Sodium hypochlorite; Contamination

1. Introduction

Ancient DNA (aDNA) can often be successfully extracted from skeletal remains and is a valuable tool for addressing questions about prehistory (for reviews see [1,2]). However, as DNA extracted from ancient remains tends to occur in low copy number and is highly degraded [3,4], the analysis of aDNA is highly susceptible to contamination originating from modern sources introduced to remains during the course of their excavation and/or study. Modern contaminating DNA competes with endogenous aDNA in polymerase chain reaction (PCR) amplification, and thus, can yield false positive and/or aberrant results. As forensic scientists often deal with limited amounts of degraded DNA in skeletal remains and other samples (e.g. hair) [5–11], they too must contend with this sensitivity of PCR to contaminating DNA [12] and, thus, utilize protocols and precautions similar to those used by ancient DNA researchers [13–15]. This paper systematically evaluates the use of bleach for decontaminating the surface of ancient bones, and is equally relevant to forensic analyses as well.

There are three sources of contamination of ancient DNA extractions: (1) co-extracted surface contamination on the bone or tooth, resulting from contact with the material at any point between the time of excavation in the field by archae-

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doi:10.1016/j.forsciint.2004.11.017

Table 1
Examples from the ancient DNA and forensics literature of studies that employed methods to address contamination found on the surfaces of bones and teeth

Study/reference	Outer layer cleaned	Removal of surface	Extraction of internal material	Acid wash	UV irradiation	EDTA presoak	Ethanol	Hydrogen peroxide	Bleach
[37]		×			×				×
[33,38]		×			×				
[8,10]	×	×							×
[39]		×					×		×
[23,40,41,39,42–45]		×							
[46,47]		×		×			×		
[48]		×	×						
[49]	×				×				×
[50–52]					×				×
[53]				×	×				×
[54,55]					×				
[56]						×			×
[57]			×				×		×
[58]	×							×	×
[31]							×	×	×
[6]							×		
[7]	×								

No consensus has emerged on the most efficient technique as demonstrated by the variation of methods used in these studies.

ologists to DNA extraction by laboratory personnel, (2) reagents, labware, and lab disposable supplies contaminated during their manufacture, packaging, and/or distribution [16], and (3) PCR carryover, the inadvertent transfer of DNA between tubes during the course of analysis. Our study addresses the first source of contamination.

A variety of methods are utilized by aDNA researchers and forensic scientists to remove or exclude contamination before DNA extraction begins, including: (1) washing the surface of the bone or tooth, (2) physically removing the surface of the bone or tooth, (3) extracting material from only the interior of the bone or tooth, (4) washing the surface of the bone or tooth with acid, (5) irradiating the bone or tooth with UV light, (6) exposing the bone or tooth to highly concentrated ethanol, (7) exposing the bone or tooth to a bleach (sodium hypochlorite—NaOCl) solution, or (8) combinations of these techniques. Table 1 describes the decontamination methods employed in 30 previous studies. While various combinations of the methods cited above were used, surface removal was the most frequently method employed, followed by exposure to bleach, then UV irradiation. The choice in applied decontamination method is likely based on the researchers' views on the nature of contamination combined with a consideration of time, cost, and the results of previous studies. For example, those researchers that chose to remove the surface likely believe that contaminant DNA does not penetrate more deeply into the bone or tooth than can be redressed by abrasion. The application of bleach is certainly cost effective and this technique is less laborious than surface removal. However, it is noted that the two techniques have been applied together in four of the studies listed in Table 1.

Sodium hypochlorite "destroys" DNA through oxidative damage, such as base modifications, and the production of chlorinated base products [17–20]. Exposure of DNA to increasingly higher concentrations of sodium hypochlorite causes cleavage of the strands, breaking the DNA into smaller and smaller pieces, and eventually to individual bases (Dr. Clare Hawkins personal communication 2004). Prince and Andrus [21] found that exposure of DNA to sodium hypochlorite precluded PCR from amplifying a 76 base pair (bp) amplicon, suggesting that the DNA had been broken down into sizes smaller than this. It is, therefore, of some concern that exposure of skeletal remains to sodium hypochlorite destroys only surface DNA, leaving the endogenous DNA intact.

While bleach has been widely employed to decontaminate skeletal remains, the concentration of bleach used, length of time the bone or tooth is exposed to bleach, and the manner in which bleach is applied to the bone or tooth varies widely. Table 2 describes the procedures employed by the 14 studies listed in Table 1 that used bleaching as a decontaminating procedure. Application methods described in Table 2 are stated as closely as possible to that reported in the original publication cited; as such, "soaked" may be the same as "immersed." All concentrations are percent (v/v) (bleach/water) except those noted with an asterisk (*), which were reported as percent sodium hypochlorite. It is assumed that all bleach solutions were diluted from household bleach, which is 5–6% (w/v) sodium hypochlorite in full strength. The "unspecified" concentration is assumed, but not known, to be full strength household bleach. It is noteworthy that the duration of exposure to bleach solution in five of the studies is "unspecified" and the concentration of bleach used is not

Table 2
A sample of studies (from Table 1) that reported the use of bleach for decontaminating bone or tooth surfaces

Study	Concentration (%)	Duration (min)	Application method
[56]	20	2	Immersion of powered bone
[39]	10	10	Immersion of teeth only (not bone)
[49]	10	10	Immersion
[58,51]	10	Unspecified	Washing
[52]	5	5	Soaked
[57]	Unspecified	5	Immersion
[37]	"Diluted"	Unspecified	Washing
[31]	"Diluted"	30	Washing and soaked
[53]	10*	10	Immersion with shaking
[50]	5*	20	Soaked
[23]	5*	1	Soaked
[8,10]	5*	Unspecified	Wipe the surface with bleach soaked cotton

specified in three of the studies. These omissions underscore the need to establish standards for effective decontamination of bone and tooth surfaces.

Prince and Andrus [21] determined that 10% (v/v) Clorox bleach (equivalent to ~0.55%, w/v, solution of sodium hypochlorite) was effective in destroying DNA, while 2.5% (v/v) Clorox bleach only caused nicking of the DNA, evidenced by slower mobility of the treated DNA during gel electrophoresis. A 20% (v/v) solution of 5.25% (w/v) sodium hypochlorite (Mr. Jason G. Linville personal communication 2002) has been shown to remove contaminating DNA from the surface of maggots, a necessary step for the forensic analysis of crop contents [22]. Richards and Sykes [23] demonstrated that bone soaked in 0.5% (w/v) sodium hypochlorite (an approximately 10%, v/v, solution of commercial bleach) was more effective in removing contamination than shotblasting the surface of the bone. To our knowledge, no systematic study has tested the effectiveness of bleach in removing surface contamination from skeletal remains. In this paper, we compare the effectiveness of various concentrations of sodium hypochlorite and exposure times for removing contaminating DNA from bones in order to optimize the use of this chemical for decontaminating purposes.

2. Materials and methods

2.1. Materials

A single rib from each of four different individuals was used in the experiments described below. The first represents an archaeological site in Butte County, California, which was occupied between 500 and 3500 years BP (Dr. Jason Eshleman personal communication). The three remaining ribs were from the Post-Classic (defined as the 10th–16th centuries A.D. in Mesoamerica) Aztec city of Tlatelolco, Mexico. These samples post-date A.D. 1325 (or A.D. 1345), the initial founding of the island city of Tenochtitlan-Tlatelolco [24], and pre-date the Contact Period (A.D. 1519). All four samples were previously assigned to the Native American mitochondrial haplogroup A based on two independent extractions. Haplogroup A is defined by the *Hae*III site gain at nucleotide (nt) 663 in the mitochondrial genome [27] according to the Cambridge reference sequence [28]. The haplogroup of the sample from Butte County, California, is unpublished (Dr. Jason Eshleman personal communication, extraction method is found in [25]) and Kemp et al. [26] reported the haplogroup assignment of the Aztec ribs.

Clorox bleach (6.0%, w/v, sodium hypochlorite) was used in all experiments and all dilutions are reported as percent (w/v) sodium hypochlorite rather than as percent (v/v) diluted bleach because the strength of commercial bleach varies.

2.2. Methods

2.2.1. Introduction of contamination

Each of the aforementioned bones was handled with bare hands by the first author (B.M.K.) for approximately 30 min preceding the sodium hypochlorite treatments (except in "sodium hypochlorite experiment five" (Section 2.2.6)), thereby introducing modern contaminating DNA onto the surface of each of the samples. The mitochondrial DNA (mtDNA) of this author belongs to haplogroup U which lacks the *Hae*III restriction site at nt 663 that is diagnostic of haplogroup A. Therefore, contamination present in the extracted DNA can be readily identified by the presence of an uncut amplicon (the product of the author's mtDNA) following digestion of the amplified extract with the *Hae*III restriction enzyme. In this case, the uncut amplicon will be accompanied by a cut amplicon, the product of the ancient DNA. When the contamination is successfully destroyed by the sodium hypochlorite treatment (described below) only the cut amplicon is present. This methodology is illustrated in Fig. 1 and is similar to that designed to assess the presence or absence of contamination in previous studies [23,22]. While we cannot know that each of the four bone samples was equally contaminated, the bones were handled with the intent of providing equal exposure to the four samples. Furthermore, we believe that this technique should introduce more con-

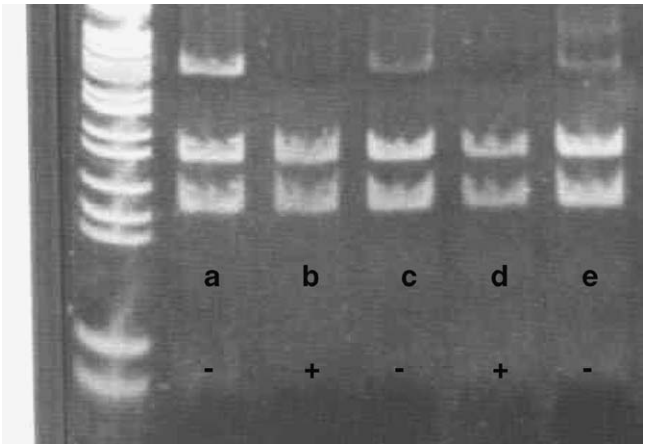

Fig. 1. Image of a polyacrylamide gel showing the results of ''sodium hypochlorite experiment one'' (Section 2.2.2) (treatments a–e). The restriction enzyme digested amplicons illustrate how the presence/absence of contamination was scored. In lanes a, c, and e, the larger amplicon represents contaminating DNA (which lacks the *Hae*III site gain) and the two smaller fragments are the product of the ancient DNA that has been cut at the *Hae*III site. Lanes b and d lack the larger amplicon and only show the presence of the two smaller fragments, products of the ancient DNA that has been cut at the *Hae*III site.

tamination to the bones than ''standard'' archaeological or forensic samples would encounter by happenstance.

2.2.2. Sodium hypochlorite experiment one

This experiment was conducted to compare the effectiveness of sodium hypochlorite with that provided by alternative methods of decontamination, specifically the removal of the bone surface and use of ''DNA Away''. The first rib (from Butte County, CA) was subdivided into five pieces of approximately equal size. One each of these five fragments was then treated in the following manner prior to DNA extraction:

(a) No treatment (to ensure contamination had been introduced).
(b) Immersion in 2% sodium hypochlorite for 10 min.
(c) Removal of the bone surface with sandpaper.
(d) Immersion for 2 min in ''DNA Away'' (Molecular BioProducts—a proprietary substance that is probably highly concentrated sodium hydroxide, based on the details of the chemical's MSDS).
(e) Immersion in 0.6% sodium hypochlorite for 10 min.

2.2.3. Sodium hypochlorite experiment two

In the context of the results of the first experiment (see Sections 3 and 4), a second experiment was conducted to assess the repeatability of these findings and further manipulate sodium hypochlorite concentration and exposure time. Part of a second (Aztec) rib was subdivided into seven fragments of approximately equal size. One each of these seven fragments was then treated in the following manner prior to DNA extraction:

(f) Immersion in 0.6% sodium hypochlorite for 15 min.
(g) Immersion in 1.2% sodium hypochlorite for 15 min.
(h) Immersion in 2.0% sodium hypochlorite for 15 min.
(i) Immersion in 3.0% sodium hypochlorite for 15 min.
(j) Immersion in 3.0% sodium hypochlorite for 30 min.
(k) Immersion in 6.0% sodium hypochlorite for 30 min.
(l) No treatment (to ensure contamination had been introduced).

2.2.4. Sodium hypochlorite experiment three

This experiment was conducted to determine the effect of extreme exposure of both contaminant and endogenous DNA to sodium hypochlorite. Each of four equally sized fragments of the second rib from ''sodium hypochlorite experiment two'' (Section 2.2.3) were treated separately as follows:

(m) Immersion in 6.0% sodium hypochlorite for 1 h.
(n) Immersion in 6.0% sodium hypochlorite for 2 h.
(o) Immersion in 6.0% sodium hypochlorite for 4.3 h.
(p) Immersion 6.0% sodium hypochlorite for 21 h.

Note that the ''no treatment'' portion of this bone was part of ''sodium hypochlorite experiment two'' (Section 2.2.3).

2.2.5. Sodium hypochlorite experiment four

This experiment was conducted to assess repeatability of the results of ''sodium hypochlorite experiment three'' (Section 2.2.4), and address any problems that ensued (see Sections 3 and 4). A portion of the third (Aztec) rib was subdivided into two fragments of equal size and each was treated separately, as follows:

(q) No treatment (to ensure contamination had been introduced).
(r) Immersion in 6.0% sodium hypochlorite for 18 h.

2.2.6. Sodium hypochlorite experiment five

This experiment was conducted to test the long-term effect of contamination on a bone surface. The fourth (Aztec) rib was handled as described in ''introduction of contamination'' (Section 2.2.1), placed in a disposable weighboat, and stored uncovered on a shelf for 14 months at room temperature. A portion of the bone was subsequently subdivided into three fragments of equal size and one each of these three fragments was treated as follows:

(s) No treatment (to ensure contamination had been introduced).
(t) Immersion in 3.0% sodium hypochlorite for 15 min.
(u) Immersion in 6.0% sodium hypochlorite for 15 min.

2.2.7. DNA extraction

DNA was extracted from the bone fragments of each set of the above-mentioned experiments at different times. In order to detect contamination generated during the extraction process, a negative control (extraction blank) was analyzed in parallel with each of the five sets of extractions. Following the respective treatments, the sodium hypochlorite solution (if used) was discarded and the bones were immersed in DNA-free ddH_2O (Gibco) for 1–2 min. After discarding the water, the bone was repeatedly rinsed with water to ensure removal of any remaining bleach. The bone fragments were transferred to 15 mL conical tubes, demineralized by adding a sufficient amount of 0.5 M EDTA (pH 8) to submerge the bones (1–4 mL), and gently rocked for at least 48 h at room temperature. Following demineralization, 3 mg of proteinase K (Gibco BRL) were added and the samples were incubated at 65 °C for 8–14 h to allow for maximum digestion of the bone proteins.

DNA was extracted from the digested samples using a three-step phenol/chloroform method: two extractions adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the EDTA and one extraction with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding two volumes of cold absolute ethanol and one half volume of cold 5 M ammonium acetate, then storing the solution at −20 °C for 8–10 h. The tubes were centrifuged to pellet the DNA, the liquid was discarded, and the pellet washed 1–4 times with 80% ethanol, each time pelleting the DNA and discarding the ethanol. The pellet was then dried at room temperature for 24 h. To remove co-extracted PCR inhibitors, the pelleted DNA was resuspended in 300 μL of ddH_2O and silica extracted [29] using the Wizard DNA Purification Kit (Promega), following the manufacturer's instructions (except that the DNA was finally eluted with 100 μL of ddH_2O).

2.2.8. PCR amplification

PCR amplification reactions contained 3 μL of DNA template, 4 μL of 5 mM dNTPs, 2.5 μL of 10× PCR buffer, 1.3 μL of bovine serum albumin (BSA), 0.75 μL $MgCl_2$, 0.3 μL of each primer (50 μM), 1.5 units of Platinum Taq (Invitrogen), and sufficient ddH_2O to adjust the reaction to a total volume of 25 μL. Coordinates for primers used, numbered according to the Cambridge Reference Sequence [28], are: nt 591–611 and 765–743 [30]. PCR conditions were as follows: 94 °C for 5 min, 40 cycles of successive 30 s holds at 94, 55, and 72 °C, followed by a final 3 min extension period at 72 °C. Negative controls (PCR blanks) accompanied all PCR reactions to detect any unintentional contamination arising in the PCR reaction set-up. Approximately 5–7 μL of amplicon were electrophoresed on 6% polyacrylamide gels and visualized with ethidium bromide to confirm the successful amplification of mtDNA. Digestion of 7–10 μL of each amplicon was performed with 8 units of *Hae*III restriction enzyme (Promega). These products were electrophoresed and visualized as described above.

3. Results

Results were recorded as either the complete removal of contamination or the lack thereof, respectively, documented by the presence of one type or two types of amplicon(s) following restriction enzyme digestion. The results for all treatments are shown in Table 3. Three of the four ''no treatment'' extracts (treatments a, l, and q) revealed the presence of contaminating DNA, while the fourth ''no treatment'' (treatment s) did not exhibit any contamination.

In ''sodium hypochlorite experiment one'' (Section 2.2.2), both the immersion in 2.0% sodium hypochlorite solution for 10 min (treatment b) and DNA Away for 2 min (treatment d) successfully removed contamination. The negative control (extraction blank) did not amplify, indicating that the extraction process did not introduce contamination.

In ''sodium hypochlorite experiment two'' (Section 2.2.3), immersion in at least 3.0% or more sodium hypochlorite solution for at least 15 min (treatments i–k) was required to remove contamination. Both ''no treatment'' (treatment l) and immersion in 1.2% sodium hypochlorite solution for 15 min (treatment g) allowed only contaminating DNA to be amplified, indicated by the sole presence of uncut amplicons following restriction enzyme digestion. The negative control (extraction blank) did not amplify, indicating that the extraction process did not introduce contamination.

In ''sodium hypochlorite experiment three'' (Section 2.2.4), which assessed extreme exposure of bone to sodium hypochlorite, none of the treatments (treatments m–p) eliminated the contamination. In this experiment, the negative control (extraction blank) also amplified, indicating that unintentional contamination had been later introduced during one of the many steps of the DNA extraction following the initial treatments of the bones.

In ''sodium hypochlorite experiment four'' (Section 2.2.5), immersion in 6.0% sodium hypochlorite solution for 18 h (treatment r) effectively removed the contamination.

Table 3
Results of the various decontamination treatments (a–u, corresponding to the letters found in the text) employed in this study

Treatment	Experiment number	NaClO or other treatment (%)	Time exposed	Contamination removed
a	1	No treatment	N/A	−
b	1	2.0	10 min	+
c	1	Removal of surface	N/A	−
d	1	DNA away	2 min	+
e	1	0.6	10 min	−
f	2	0.6	15 min	−
g	2	1.2	15 min	−#
h	2	2.0	15 min	−
I	2	3.0	15 min	+
j	2	3.0	30 min	+
k	2	6.0	30 min	+
l	2	No treatment	N/A	−#
m	3	6.0	1 h	−*
n	3	6.0	2 h	−*
o	3	6.0	4.3 h	−*
p	3	6.0	21 h	−*
q	4	No treatment	N/A	−
r	4	6.0	18 h	+
s	5	No treatment	N/A	+
t	5	3.0	15 min	+
u	5	6.0	15 min	+

Removal of contamination was confirmed by the presence of only cut amplicons following restriction enzyme digest (Fig. 1), and is denoted by a plus symbol (+). When contamination was not completely destroyed we denote these results with negative symbols (−). The pound symbol (#) indicates that only contaminating DNA amplified, that is, the contamination entirely outcompeted the ancient DNA in PCR amplification. An asterisk (*) indicates the presence of two types of DNA following restriction enzyme digest as a result of a contaminated extraction procedure following initial treatment.

The negative control (extraction blank) did not amplify, indicating that the extraction process did not introduce contamination.

In ''sodium hypochlorite experiment five'' (Section 2.2.6), neither sodium hypochlorite treatment revealed the presence of contamination. However, despite following the same protocol as used in each of the other experiments to ensure successful introduction of contamination to the bone surface, the sample untreated by sodium hypochlorite (treatment s) also revealed no evidence of contamination.

4. Discussion

This study demonstrates that sodium hypochlorite, when applied correctly, can be used as an effective means of destroying DNA contamination on bone surfaces. The extraction and amplification of contaminant modern mtDNA from three untreated prehistoric bones (treatments a, l, and q) illustrates that modern contamination can be introduced on the surface of a bone or tooth through handling and that it competes with the endogenous ancient DNA in PCR amplification. The results from ''sodium hypochlorite experiment two'' (Section 2.2.1), in which two of the treatments (g and l) produced only contaminating DNA amplicons in the PCR reaction, are of particular interest because they demonstrate that the contamination extracted from a bone surface can entirely outcompete aDNA in PCR amplification. These results suggest that to preclude false positives and aberrant results from the analysis of samples containing degraded DNA, one must absolutely remove any exogenous contamination before proceeding with DNA extraction. These results could also be interpreted as the failure of the extraction method employed to isolate the endogenous ancient DNA in these two cases. However, in later rounds of amplification and enzymatic digestion, the presence of the endogenous aDNA in the extracts studied in ''sodium hypochlorite experiment two'' (Section 2.2.3) was confirmed. Thus, the stochastic nature of PCR amplification can produce variable results, even when identical protocols are used, underscoring the importance of repeated trials.

The results of ''sodium hypochlorite experiment one'' (Section 2.2.2) suggested that bone must be immersed in at least a 2% sodium hypochlorite solution for 10 min (treatment b) or in DNA Away for 2 min (treatment d) to remove contamination from its surface. We did not further experiment with DNA Away, as it is a more expensive and not a demonstrably more effective reagent than bleach. It is interesting to note that physically removing the surface of the bone (treatment c) did not remove the contamination, suggesting that contaminant DNA can penetrate more deeply than can be redressed by its removal by abrasion or excision. Therefore, we strongly suggest the use of bleach as the most cost-effective means for decontaminating bone surfaces,

further avoiding the necessity of removing the surface of the bone, which can be laborious and messy.

Some of the results of ''sodium hypochlorite experiment two'' (Section 2.2.3) appear to contradict those of ''sodium hypochlorite experiment one'' (Section 2.2.2). In the latter experiment, the immersion of a sample in 2.0% sodium hypochlorite for 10 min (treatment b) destroyed the contamination, while in the former experiment immersion in 2% sodium hypochlorite for a longer period of time (15 min) failed to remove contamination. In ''sodium hypochlorite experiment two'' (Section 2.2.3) immersion in 3% sodium hypochlorite for at least 15 min was required to eliminate contamination. These conflicting results may be a consequence of inadvertently depositing more contamination onto the surface of the first Aztec rib than onto the surface of the Butte County, California, sample. It should be noted that although aDNA and forensic studies have generally employed less concentrated sodium hypochlorite solutions for decontaminating samples (Table 2), they probably encountered lesser amounts of contamination (if present at all) than employed in the present study. That is, our introduction of contamination (handling the bones for 30 min) may have introduced more contamination to the bones than ''standard'' archaeological or forensic samples would encounter by happenstance. It is likely that varying levels of contaminating DNA on forensic or archaeological skeletal remains will require the use of varying concentrations and time of exposure to sodium hypochlorite. Thus, we recommend the protocol cited above as a conservative precautionary method that can confidently destroy contamination from extreme exposure to modern DNA.

The results of ''sodium hypochlorite experiment three'' (Section 2.2.6) were compromised by unintentional contamination that occurred during the extraction procedure itself, following the initial treatments of the bone fragments. These results draw attention to the importance of close monitoring of contamination and demonstrating the repeatability of results when working with degraded DNA. However, we believe that if this extraction had not become contaminated all four treatments (of 6% sodium hypochlorite for greater than 1 h) would have removed the contamination because in ''sodium hypochlorite extraction two'' (Section 2.2.3) all treatments of at least 3% sodium hypochlorite successfully destroyed the contaminant DNA. Since the bone used in this experiment is the same as that in ''sodium hypochlorite experiment two'' (Section 2.2.3), differing amounts of contamination on the bone cannot explain these results. The results of ''sodium hypochlorite experiment four'' (Section 2.2.5) confirm that contaminant DNA is destroyed by immersion in 6% sodium hypochlorite for 18 h.

Most striking about the results of ''sodium hypochlorite experiment three'' (Section 2.2.4) and ''sodium hypochlorite experiment four'' (Section 2.2.5) is that the integrity of the ancient DNA was not compromised by such extreme sodium hypochlorite treatments. This is evidenced by the detection of the presence of the *Hae*III 663 restriction site, diagnostic of mitochondrial haplogroup A, in extracts of treatments m–p, and r. We believe this is indirect support of the hypothesis that DNA binds to hydroxyapatite ($Ca_5(PO_4)_3OH$), crystalline calcium phosphate that comprises the bulk of the bone's matrix, and that this reaction aids in the preservation of DNA in ancient skeletal remains [31–34]. While the mechanism of this reaction is uncertain, the negatively charged phosphate groups in the backbone of the DNA molecule probably bind to hydroxyl sites on the hydroxyapatite ([35] cited in [34]). Consistent with this hypothesis, Götherström et al. [34] noted a negative relationship between the preservation of DNA and increased crystallinity (degradation) of hydroxyapatite and a positive relationship between the preservation of collagen and DNA. Since the strong binding of collagen and non-collagenous proteins to hydroxyapatite prevents their degradation by temperature and chemical agents [34,36], the biding of DNA to hydroxyapatite might prevent its oxidative damage by even extreme treatments of sodium hypochlorite (immersion in up to 6% solutions for 21 h in the present study).

The results of ''sodium hypochlorite experiment five'' (Section 2.2.5) are intriguing because contamination was absent from the surface of the non-treated bone that had been left out for 14 months (treatment s). In this case, the two sodium hypochlorite treatments (treatments t and u) were not responsible for removal of contamination; during the 14-month time period between handling the bone and extracting DNA from it, contamination was apparently eliminated by ''natural'' forces. These results suggest that contaminating DNA on the surface of a bone is not protected from degradation as is the endogenous DNA, further supporting the notion that the hydroxyapatite in the bone matrix protects against the chemical degradation of DNA. An alternative explanation which, unfortunately, was not eliminated from consideration by the immediate confirmation of contamination introduced onto the bone surface, is that, the initial handling did not successfully contaminate the bone. In support of our argument here, however, it should be noted that in every other case of handling (treatments a, l, and q) the bone was successfully contaminated, as intended. The ''natural'' removal of contamination from this bone might suggest that all of the experiments conducted in this study are superfluous because with sufficient time decontamination procedures are unnecessary. However, as clearly demonstrated by the rigorous contamination removal employed by previous studies (Table 1) and the majority of the results present here, contamination does occur when working with degraded samples, and it must be removed before DNA extraction is initiated. Sufficient time to allow ''natural'' decontamination to occur in a DNA-free environment is likely not to be available in most forensic and scientific contexts.

As a result of these experiments, we now regularly immerse both bones and teeth in 6.0% sodium hypochlorite for 15 min prior to DNA extraction and have found this to be the most practical and cost-effective technique for removal of contamination from samples ranging between 500 and 10,000 years old [26] and unpublished data by Kemp et al.

Acknowledgments

Cara Monroe, Dr. Ripan S. Malhi, and Dr. Jason Eshleman for helpful comments on the experimental design and writing of the paper. Dr. Clare Hawkins for aiding us in understanding radical biology. This work was supported by NIH grant RR00169, a faculty research grant from the regents of the University of California, a dissertation grant from UC Mexus, and an individual research grant from Wenner Gren.

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