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Further evaluation of the efficacy of contamination removal from bone surfaces

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ABSTRACT

Studies of low copy number (LCN) and degraded DNA are prone to contamination from exogenous DNA sources that in some cases out-compete endogenous DNA in PCR amplification, thus leading to false positives and/or aberrant results. Particularly problematic is contamination that is inadvertently deposited on the surfaces of bones through direct handling. Whereas some previous studies have shown that contamination removal is possible by subjecting samples to sodium hypochlorite prior to DNA extraction, others caution that such treatment can destroy a majority of the molecules endogenous to the sample. To further explore this topic, we experimentally contaminated ancient northern fur seal (*Callorhinus ursinus*) ribs with human DNA and treated them with sodium hypochlorite to remove that contamination. Our findings are consistent with previous studies that found sodium hypochlorite to be highly efficient (~81–99%) at contamination removal; however, there emerged no treatment capable of removing 100% of the contamination across all of the experiments. Moreover, the ability to estimate the degree of damage to endogenous northern fur seal molecules was compromised due to the inherent variability of preserved mtDNA across the bones, and the presence of co-extracted PCR inhibitors.

1. Introduction

The advent of the polymerase chain reaction (PCR) [1] revolutionized the fields of forensic and ancient DNA (aDNA) studies [e.g., 2–6]. Subsequent advancements in PCR technology and chemistry have allowed DNA amplification from increasingly minute amounts of template molecules. While PCR now permits the routine study of genetic markers contained in degraded samples, it simultaneously represents a system that is hypersensitive to amplifying contaminant DNA [7–9].

Tempering the strength of DNA evidence collected from degraded remains and other aged biological materials is its challenging retrieval and authentication, principally because of the damage that the molecules have undergone since the death of the individual or the deposition of the biological material. Degradation by nucleases, oxidation, hydrolysis, deamination, and depurination lead to destabilization, breaks, and chemical modifications of DNA strands, leaving DNA template molecules that are few in number, typically short in length and carry "damaged" nucleotide positions [2,3,10–12]. As a result, studies of low copy number (LCN) and degraded DNA are prone to contamination from exogenous DNA sources that in some cases can out-compete endogenous DNA in PCR amplification, thus leading to false positives and/or aberrant results [e.g., 13].

There are four ways that contaminating DNA may be introduced into a study: (1) DNA introduced in the field (e.g., through handling), (2) DNA introduced by laboratory personnel, (3) cross contamination between samples and/or PCR products and samples, and (4) DNA present in pre-packaged laboratory reagents and/or present on labware. Addressing the second and third sources of contamination is generally an issue of maintaining high standards within the laboratory, so it is not surprising that forensic DNA researchers, and others working with LCN and degraded DNA samples largely agree on a common set of practices [14–16]. Purchasing goods and reagents guaranteed to be DNA-free can aid in minimizing the fourth source of contamination, but regardless of an awareness of the first source of contamination [17–19], contaminating DNA can be inadvertently deposited on the surfaces of bones and teeth through handling, from contact with other bodily fluids (e.g., perspiration or saliva), or when a specimen

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comes into contact with another contaminated object. In aDNA studies this source of contamination often enters the study during archeological excavation and analyses [18]. In forensic investigations, crime labs are often presented with human remains that have poor provenience and have been directly handled [20]. In general, forensic researchers are aware of the problems that contamination poses, however, law enforcement officers, morticians, and pathologists may be less informed [21–25].

If contamination is detected, determining its source can be time consuming, expensive, and/or impossible when, for example, comparative DNA profiles of law enforcement officers and others involved with a case are nonexistent [23]. It is compelling, therefore, to develop a method that can remove contaminating DNA, while simultaneously having an insignificant effect on the endogenous target DNA. Achieving this goal could reduce the cost of future studies, make them less labor-intensive, and strengthen the weight of evidence gathered from degraded sources.

Subjecting bones and teeth to sodium hypochlorite (NaOCl or bleach) is one of the most common methods used for contamination removal [see studies reviewed by 13, 26]. Both aDNA researchers and forensic scientists have conducted experiments aimed at evaluating methods of contamination removal; some conclude that it is difficult to entirely remove the contaminants [27–30], while others show that complete decontamination is possible [13,31,32]. For example, Kemp and Smith [13] demonstrated that contaminating human mitochondrial DNA (mtDNA), experimentally deposited on the surfaces of ancient human bones, could *only* be removed by submersion in $\geq$3.0% sodium hypochlorite[1] for 15 min. In contrast, studies by Malmström et al. [28,29] and Gilbert et al. [27], utilizing 0.5–3.0% sodium hypochlorite, failed to sufficiently remove contamination.

The ability to amplify DNA from a bone or tooth after any treatment with sodium hypochlorite suggests that the endogenous DNA is protected from this heavy oxidant through its adsorption to hydroxyapatite, a bond not afforded to the contaminating DNA [26]. Kemp and Smith [13] demonstrated that endogenous mtDNA was still recoverable from a bone fragment submerged in 6.0% sodium hypochlorite for 21 h, however, they did not evaluate the extent of degradation, if any, suffered by the endogenous DNA during experimental treatments. Dissing and colleagues [31] have shown that radioactively labeled hypochlorite (ClO^- containing the Cl^{36} isotope) migrates into the pulp of teeth after 30 min of submersion, suggesting, but not demonstrating, that bleach treatment could potentially destroy some of the endogenous DNA within a specimen. Utilizing quantitative PCR (qPCR), Malmström et al. [29] argued that the amount of authentic aDNA is reduced by 77% when *powdered* bone or tooth is subjected to sodium hypochlorite before extraction.

To our knowledge, no investigations have evaluated the effect of bleach on endogenous DNA when whole bone fragments or teeth are treated for contamination. Here the efficacy of contamination removal from whole bone pieces by various treatments with bleach is measured in parallel to the effects that these treatments have on endogenous target DNA.

2. Materials and methods

2.1. Samples

The northern fur seal (*Callorhinus ursinus*) ribs studied were excavated from the Amaknak Bridge Site in Unalaska, AK and date to approximately 3500 years before present (YBP) [33]. They have an unknown handling history since excavation and have previously been determined to contain variable amounts of preserved mtDNA [34,35]. Segments of the ribs that remained after the study of Barta et al. [35] were used in these experiments. These segments of bone span: (1) from the proximal pieces removed to the middle portions removed (here these remaining segments are called "proximal segments") and (2) from the middle portions removed to the distal portion removed (here these remaining segments are called "distal segments").

2.2. Experimental contamination

Each segment was bare-hands handled by the 3rd author of the study for 20 min, rotating the bone in his hand with the intention to thoroughly and evenly introduce contaminating human mtDNA across the bone. Following this treatment, the segments were sealed in a plastic bag, immediately given to the 1st author of the study, and further processed within 24 h. Cross-sections of bones were removed perpendicular to the length of ribs working in the direction from proximal to distal using a new Dremel® rotary blade for each cross-section removed. This work was conducted under a fume hood in the geoarchaeological laboratory at Washington State University, which is located in a separate building from the post-PCR facility. The inside surfaces of fume hood were wiped down with ~1.5% sodium hypochlorite before and after use. The first cross-section removed was intentionally not processed further because throughout the bare-hands handling procedure, the ends of the segments were also subjected to the introduction of contamination, so that the first cross-section might have been more contaminated because of its greater surface area exposed to contamination compared to the subsequent cross-sections removed. This was not an issue for the last cross-sections removed, as they did not include the distal end of the segments.

2.3. Experimental treatments

All subsequent preparation methods (i.e., experimental decontamination, DNA extraction and PCR set-up) were conducted in the aDNA laboratory at Washington State University, one dedicated to the analysis of degraded and low copy number (LCN) DNA. Appropriate measures to minimize contamination and, importantly, to detect it if present, were employed [16].

Cross-sections removed from seven rib segments [809038 and 809039 proximal segments, and 809007, 809021, 809039, 809046 and 809053 distal segments (for the remainder of this paper these segments will simply be referred to as: 809007D, 809021D, 809038P, 809039P, 809039D, 809046D, and 809053D)] were examined in this study. Each portion of bone removed from the whole was weighed and cross section photographs were taken with an accompanying scale. The scaled photographs were imported into ImageJ 1.43 s [36], and the total area of each cross section was recorded as the average of three measurements taken (i.e., by tracing around the perimeter of the cross section). The thickness of each cross section was estimated by taking the average of 3–8 measurements with digital calipers. From this, the "density index" of each cross-section was calculated following Barta et al. [35] by dividing the volume (cross section area estimated from the photograph times the average thickness taken with calipers) of each cross-section by its weight (Table 1).

Cross-sections taken from across each rib segment were subsequently treated, prior to DNA extraction, as follows:

1. No treatment 1.
2. Submersion in 30 mL of 6.0% sodium hypochlorite for 15 min.
3. Submersion in 30 mL of 4.8% sodium hypochlorite for 15 min.
4. Submersion in 30 mL of 3.6% sodium hypochlorite for 15 min.
5. No treatment 2.
6. Submersion in 30 mL of 3.0% sodium hypochlorite for 15 min.
7. Submersion in 30 mL of 1.5% sodium hypochlorite for 15 min.
8. Submersion in 30 mL of 0.6% sodium hypochlorite for 15 min.
9. Submersion in 30 mL of water for 15 min [with the exception of sample 809021D].
10. No treatment 3.

2.4. DNA extraction and quantification

DNA was extracted from the samples in batches, according to their segment, following Kemp et al. [37]. One or two extraction blanks accompanied each batch of extractions to monitor contamination in reagents.

Quantification by real time PCR was conducted with Applied Biosystems 7300 system with two different primer sets and three different probes. First, a 181 base pair (bp) portion of the cytochrome B gene of the northern fur seal mitochondrial genome was amplified and quantified following Winters et al. [34].

Second, a 149 bp portion of the first hypervariable region (HVRI) of the human mitochondrial genome was amplified with primers 15986F and 16153R [37] and quantified with two separate probes in two separate reactions. The first of these, designed to count all human DNA, was a MAR labeled probe 5′-GACTCACCCATCAACAACC-3′ (Allelogic). This probe corresponds to nucleotide

[1] Many researchers inconsistently report their usage of bleach, leading to confusion about what has been employed [see discussion of this by 13]. In this paper, all percentages of bleach represent percent of sodium hypochlorite (w/v). In this case 3% sodium hypochlorite is equivalent to a 1:1 dilution of full strength Clorox beach (6% sodium hypochlorite) to water.