

TECHNICAL NOTE

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The Effects of Skeletal Preparation Techniques on DNA from Human and Non-Human Bone

ABSTRACT: The forensic pathologist increasingly relies on the forensic anthropologist to be the consulting expert in human identification. Likewise, if identification is not possible from visual inspection of skeletal remains, the forensic biologist may be called upon to conduct DNA analysis. The possibility of downstream DNA testing needs to be considered when skeletal preparation techniques are employed to deflesh human remains, as they have the potential to strongly impact genetic analyses and subsequent identification. In this study, three cleaning techniques, boiling bone in water, in bleach, and in powdered detergent/sodium carbonate, were tested for their effect on nuclear and mtDNA recovery from a variety of human and non-human bones. A statistically significant reduction in DNA yields occurred in non-human bones cleaned with bleach, and DNA degradation was apparent electrophoretically. The human bones also showed much lower yields from bleach cleaning, while the detergent/carbonate method allowed the largest segments of DNA to be amplified, indicating it may have a less degradative effect on bone DNA than either of the other cleaning processes.

KEYWORDS: forensic science, mitochondrial DNA, forensic biology, forensic anthropology, skeletal preparation, bone, bleach, degradation

Forensic anthropologists increasingly consult on fleshed human remains cases in which the examination of skeletal elements is critical in answering questions of identification and the circumstances of death. In such cases, fast, safe, and inexpensive methods for defleshing human remains are desirable. Many techniques have been introduced for quickly preparing skeletal material, including cooking or maceration in water or chemicals (1). Not surprisingly the chemicals used, while accelerating the defleshing process, are quite harsh, potentially dangerous, and can easily cause damage to the bone itself. Foremost among these are oxidizing agents such as household bleach (sodium hypochlorite), which while effective, often cause undesirable flaking of the bone surface and potential loss of evidentiary detail. In response to this, Fenton et al. (2) recently published a detergent/carbonate method for defleshing human remains based on cooking them in a combination of powdered detergent (Alconox (Alconox Inc., White Plains NY) or various laundry detergents) and powdered sodium carbonate (available from chemical companies or as a laundry booster). The technique is safe, effective, and inexpensive, and leaves the skeletal material in a better macroscopic condition than does cooking in bleach.

In cases where the forensic anthropologist or odontologist cannot facilitate an identification of human remains, such as unavailable medical/dental records or incomplete or damaged material, forensic biologists often perform comparative DNA analyses. As genetic methodologies become increasingly used for identification

of skeletal material, it is incumbent on the forensic anthropologist and pathologist to take into account how bone preparation techniques might affect subsequent DNA analyses, and be mindful that the evidentiary value of bone exists not only at the macroscopic level, but at the molecular level as well. In other words, it is vital that skeletal preparation techniques not be deleterious to DNA. This paper presents a study of the effects of three skeletal preparation techniques on DNA yield and degradation from human and non-human bone.

Materials and Methods

More than 120 bone DNA isolations were conducted during the course of this study, both for perfecting procedures and for side-by-side comparisons, using domestic animal and human remains. Fresh femur and/or rib samples of cow (*Bos taurus*), sheep (*Ovis aries*), and pig (*Sus domesticus*), with adhering soft tissue, were obtained locally. The ribs and femurs were cut into pieces 3 to 5 cm long using a Stryker 810 Autopsy Saw (Stryker, Kalamazoo, MI). Human bone samples included three 1 cm wedges of femur, as well as 3 similarly sized bones from the foot (2 metatarsals and 1 phalange). These were obtained through standard operating procedures during the forensic investigation of a highly degraded and unidentified body discovered a few weeks post mortem. All bones sections were randomly assigned to boiling cleaning treatments.

Three cleaning solutions were tested: a control of purified water ('water'); a 25% household bleach (3% sodium hypochlorite) solution in water ('bleach'); and 20 cc of powdered Alconox and 20 cc of sodium carbonate in 2 L of water ('detergent/carbonate') according to (2). In all cases the water had been softened and then reverse osmosis treated (resistance of 10 M Ω). Bone samples were submerged, and each solution was brought to a boil and kept just

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below a rolling boil for 4 hours. Following cleanings the bones were air-dried.

Cleaned bones were cut into smaller pieces for DNA processing. Fragments of rib approximately 0.5 cm long were sliced off the end of the bone using a Dremel rotary tool (Dremel, Mount Prospect, IL). Non-human femur samples were fragmented manually (placed in a new plastic bag and struck with a hammer) until pieces of approximately 1 cm³ were obtained. A similarly sized fragment of human femur or center shaft of the foot bones was removed using the Dremel tool. Bone fragments were ground using an IKA Works A11 Basic Mill, (IKA, Wilmington, NC). Alternatively, human bone was drilled using a 1/16th inch drill bit, collecting the resultant powder. Between 0.1 and 0.2 g of bone powder was used for each DNA extraction.

DNA Extraction

DNA was isolated from the bone samples using 500 μ L of digestion buffer (50 mM EDTA, 0.5% SDS, 20 mM Tris pH 8) and 5 μ L of 20 mg/mL proteinase K at 56°C overnight. The samples were extracted twice with phenol and once with chloroform. The aqueous layer was precipitated by adding twice the volume of 95% ethanol and one-tenth the volume of 3 M sodium acetate, placed at -20°C for 1 hour, and then centrifuged at 14,000 rpm for 15 min and the pellet vacuum dried. Alternatively the aqueous layer was placed onto a Microcon-100 filter (Millipore, Beverly, MA) and centrifuged at 500 X g until only a few microliters remained. Microcon filtering was repeated twice by adding 300 μ L of 10 mM Tris pH 7.5, 1 mM EDTA (TE). In all instances the resulting DNA was brought to a final volume of 1 μ L of TE/10 mg of bone powder.

DNA Quantity and Quality

Thirty-six procedurally identical DNA isolations (four isolations from each non-human species using the three cleaning techniques, or twelve comparisons/technique) were undertaken to produce an objective measure of the cleaning methods' effects. Total DNA yields were measured by UV spectrophotometry; 2 μ L of DNA was diluted to 100 μ L using TE. The absorbencies at 260 nm and 280 nm were taken on a Beckman (Fullerton, CA) DU 520 spectrophotometer. DNA yields following each cleaning technique were compared within bone types and species using a paired T-test (3); this compensated for differences in DNA yields among bone types and species. The more degraded human bones did not produce enough DNA for spectrophotometric measurement, so real time PCR analysis was used to estimate relative mitochondrial DNA (mtDNA) yields. Two different DNA preparations of the human samples were analyzed in multiple runs on an ABI 7700 (Foster City, CA), in replicates of 3 or 5. Conditions and oligonucleotides are described below. The DNA quality (level of degradation) of non-human DNA was examined through 1% agarose gel electrophoresis of 100 ng of DNA (based on OD values). The gels were stained with ethidium bromide, visualized using UV light, and digitally photographed. Quality of the human DNA was examined by amplifying progressively larger fragments of mtDNA (see below).

DNA Amplification

A ~600 base pair (variable among species) segment of mtDNA was PCR amplified in 10 μ L volumes. Universal primers for mammals—TGAATTGGAGGACAACCAGT (forward) in the threonine tRNA gene, and CCTGAAGTAGGAACCAGATG (re-

verse) in the control region; (4, and references therein)—were used for the cow, sheep, and pig samples, while a variety of primer sets in the human mtDNA control region, including F16190–R16410 (220 bp), F15989–R16410 (421 bp), F16190–R484 (863 bp), F15989–R484 (1064 bp) (5), were used to amplify the human samples. Non-human PCR reactions contained 1 μ L of starting DNA; the human bone samples were amplified using 1 μ L (foot bones) or 0.5 μ L (femur) of DNA. All PCR reactions used cycling parameters of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 56°C for 1 min, 72°C for 1 min, and then 72°C for 5 min.

Real time PCR reactions contained a human mtDNA control region forward primer of ACCATCCTCCGTGAAATCAA and the universal reverse primer described above, which amplify a 118 bp region of HV1 (the same region was used for the quantitation standard). The 5' FAM/3' TAMAR-labeled TaqMan probe internal to this region was CCTCGCTCCGGGCCATAAC. Fifty cycles were run on an ABI 7700 using the machine's default parameters. TaqMan Universal PCR Master Mix (Perkin Elmer; Foster City, CA) was used according to the manufacturer's instruction, while reducing the amplification volume to 10 μ L.

Statistical Analyses

DNA yields can (and did; see Results) vary greatly among bone type and species, thus it was not possible to simply combine results from each treatment to determine its influence on DNA yield. Instead, a paired T-test was used to compare the results of each cleaning technique within a bone sample of the same (non-human) species.

The median difference in DNA yields between cleaning techniques could be estimated using log transformation, which corrects for data that differ over orders of magnitude. The ratio of each pair of treatments of a given bone sample (bleach versus water; bleach versus detergent/carbonate; detergent/carbonate versus water) was log transformed, and the mean ratio for all bones determined. This value was then back transformed to give the median value on the original scale.

Results

The post-cleaning appearance of the bones, both human and non-human, differed based on the cleaning technique used: water, bleach, or detergent/carbonate. Samples boiled in bleach were a brighter white than the more beige color of those boiled in water or detergent/carbonate. Also the outer layer of the samples cleaned in bleach flaked off easily, whereas the other samples had a more solid composition. In contrast there were no visible differences between samples boiled in water and those boiled in detergent/carbonate, although the latter were generally less greasy feeling.

DNA from the non-human bones organically extracted and ethanol precipitated generated low 260/280 ratios, often around 1.2, making DNA concentration estimates difficult. When Microcon filtration was used, the average OD ratios fell more in line with those expected for nucleic acids (above 1.6) hence this method was used for subsequent quantitative comparisons. OD ratios were not found to be bone type or cleaning technique related. In no instance was enough DNA obtained from the human samples for UV spectrophotometric analysis, although PCR-based analyses were informative, as discussed below.

DNA yields varied greatly among species and bone type, ranging from over 1 ng/10 mg of bone (a pig rib sample boiled in water), to just over 0.0025 ng/10 mg of bone (a sheep femur sample boiled

in bleach). In general, the pig rib samples gave slightly higher DNA yields than other bones, but there was no clear cut species or bone type that consistently gave the highest yields, and results among bones/species often ranged over orders of magnitude. This made averaging the values for each cleaning method inappropriate. Instead, a paired T-test was used to compare the results of each cleaning technique within bones of the same (non-human) species, which helped to focus on the change in DNA yield caused by each cleaning technique.

Yield differences were most notable when, controlling for species and bone type, bones cleaned with bleach were compared to the detergent/carbonate method or the water control. Bleach cleaning resulted in a statistically significant loss of DNA when compared to both water and detergent/carbonate ($p = 0.023$ and 0.042 respectively). On the other hand, there was no statistical difference in DNA yields from water and detergent/carbonate treated samples. In the twelve direct comparisons of water to detergent/carbonate (four comparisons/species), three trials generated very similar yield results (10% difference or less), two trials resulted in higher yields from the detergent/carbonate samples, and seven trials resulted in higher yield from the water samples.

An estimate of the relative difference in DNA yield between cleaning methods was made by taking the ratio of DNA yields for a given bone sample (e.g., bleach cleaning/water cleaning in a cow femur trial), log transforming the ratio, obtaining an average value for all ratios between cleaning techniques, and back transforming these data to generate a median ratio. Bleach cleaning resulted in a substantial reduction in DNA yields, generating 48% of that obtained in water cleanings, and 56% of that obtained in detergent/carbonate cleanings. The latter technique's median yield was 86% that of water in the animal samples.

Electrophoretic examination of DNAs indicated that the reduction in DNA yields of the bleach cleaning method came from direct degradation of the DNA. An example is shown in Fig. 1, where



FIG. 1—One and one half percent agarose gel electrophoresis of DNA isolated from a cleaning trial of sheep femur. Lanes 1 – 3 contain 100 ng of DNA (based on OD readings) from bones cooked in water, bleach, and detergent/carbonate respectively. Lane 4 contains a size standard (lambda DNA digested with Hind III), in which the largest band is ~23 kb and the smallest is ~0.56 kb. Note the decreased amount of high molecular weight DNA in the bleach-processed sample, both in the gel and remaining in the well.

100 ng of DNA (based on OD values) from a cleaned sheep femur was separated on a 1.5% agarose gel. Lane 2 (the bleached sample) displays an overall lower molecular weight product than those cleaned using water or detergent/carbonate. It should be noted however that it was still possible to amplify mtDNA from all cleaned samples using the mammalian conserved primers, hence complete degradation of the DNA in bleached bones did not occur.

Results from the more degraded human bones followed a pattern similar to the animal material, in that bleach treatment led to DNA loss, with perhaps a more striking advantage for the detergent/carbonate method. The real time PCR data showed that the highest mtDNA yields from both femur and foot bones were obtained after detergent/carbonate cleaning (2350 and 1150 DNA copies/ μL respectively) followed by water cleaning (550 and 440 copies/ μL), and lastly by bleach cleaning (11 and 21 copies/ μL). Sample sizes were not large enough for statistical analysis of these data, but the same pattern was seen in multiple runs. In amplifying progressively larger regions of mtDNA the detergent/carbonate method also allowed the largest region to be amplified from both bone types (1064 bp), while the water and bleach methods were successful only to 863 bp, indicating that cleaning in detergent/carbonate had a less detrimental effect on DNA. Nuclear DNA (200 and 400 bp fragments of the amelogenin gene) could not be reliably amplified from any of the cleaned human bone.

Discussion

Today it is not uncommon for forensic pathologists, morgue assistants, or anthropologists to clean skeletal material harboring putrefying material by boiling it in a bleach solution, which removes adhering soft tissue. While bleach is highly effective for defleshing bones, its drawbacks are far too great for most forensic applications, as it acts to degrade bone through decalcification and/or destruction of connective proteins and often results in visible deterioration, with outer layers readily removed as fine dust or flakes. It is not clear however how far into bone the bleach penetrates, which may itself be dependent on bone type and condition. It is also not clear the extent to which bleach deleteriously affects DNA located within skeletal material, but as DNA analysis is increasingly used for identification of skeletal remains, it is important that anthropologists and pathologists consider how bone preparation techniques might influence subsequent genetic analyses. This is particularly true of cleaning methods that utilize oxidizing agents like bleach, or acidic conditions, both of which have well documented negative consequences for DNA.

Because of the effects of bleach on bone, as well as the drawbacks of using large volumes of such a caustic substance, other cleaning methods have been developed that are equally inexpensive, as fast, and safer, including the detergent/carbonate method tested here. Beyond not causing visual damage to the bone, the detergent/carbonate solution does not contain ingredients that would be predicted to damage DNA. Indeed, that is exactly what the experiments presented here indicate. The fresh (non-human) bones cleaned in bleach showed a statistically significant reduction in DNA yield compared to both water and detergent/carbonate cleaned samples. In contrast, there was no statistical difference in DNA yields between water and detergent/carbonate cleaning, and in 5 of the 12 trials, the detergent/carbonate solution generated DNA yields equal to or higher than the water control. This indicates that unlike bleach, treatment with detergent/carbonate has no deleterious effect on the DNA yields in cleaned bones.

Gel electrophoresis revealed that much of the high molecular weight DNA retained in water and detergent/carbonate cleaned

bones was not seen in bleached bones, demonstrating that the reduced yield observed in these samples was due to DNA degradation. It was still possible to amplify DNA from the bleached samples however, meaning that the DNA was not completely degraded during the 4 h boiling process. The same was true of the human forensic samples: it was possible to amplify mtDNA from bones cleaned using all three methods. However, real time PCR showed that bleach cleaning greatly reduced mtDNA yields, by one or even two orders of magnitude. There were also indications that detergent/carbonate cleaning is preferable to even water, as only bones cleaned in detergent/carbonate generated the largest mtDNA amplicon (1064 bp). It is possible that the carbonate in the cleaning solution acts as a buffer, helping to maintain a non-acidic pH as the cooking temperature is raised, thus protecting the DNA. If this is the case it would constitute yet one more advantage of this simple cleaning technique.

In conclusion, while DNA could be obtained and amplified from both human and non-human skeletal material cleaned using any of the three methods examined in this study, it became quite apparent that a substantial reduction in DNA, due to direct degradation, occurs during bleach cleaning. The consequences of this, particularly for remains that might already be in a highly degraded state, may be extremely important when genetic analyses are conducted. In contrast, there appear to be ways to clean skeletal remains, such as the detergent/carbonate method used here, that are no more harmful

to DNA than a simple water-based process, while being much more effective in removing adhering soft tissue and grease.

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