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MINIMALLY INVASIVE HUMAN BONE. EXTRACTION METHOD FOR DNA ANALYSIS

Victoria Gibbon
University of the Witwatersrand, Parktown, South Africa

Introduction

Human skeletal material found in archaeological localities provides a wealth of bio-cultural data on the individuals and populations represented. This data was until recently collected exclusively through the traditional methods of physical anthropology. In the 1980s, the amount and variety of information dramatically increased along with the new techniques in molecular biology, which allowed the analysis of genetic material (DNA) from deceased organisms (Kaestle and Horsburgh 2002). Bone is often considered an optimal source of DNA, due to the binding of DNA to hydroxyapatite (a calcium phosphate), which slows DNA degradation over time (O'Rouke et al. 2000). Due to the compact structure of bones, DNA is usually less degraded after a period of time compared to soft tissue, which undergoes rapid degeneration (Wurmb-Scwark et al. 2003). Skeletal material is also relatively abundant in museums and university collections, which provides much material for sampling.

Historically, DNA extraction from skeletal remains has been detrimental to the holistic value of the specimen (O'Rouke et al. 2000, Kaestle and Horsburgh 2002). Large sections of bone were removed and destroyed, often leaving behind a mutilated skeleton with missing elements or sections. This obviously limits the subsequent use of the specimen for anthropological analysis, which relies on the detailed examination of the skeleton in order to reconstruct the life history of an individual. Those skeletal elements commonly used for DNA extraction are the femur (Hagelberg et al. 1991, Cattaneo et al. 1995, Kolman & Tuross 2000, Wurmb-Scwark et al. 2003, Gilbert et al. 2005), ribs (Kemp and Smith 2005), vertebrae (Cattaneo et al. 1995), and teeth (Meyer et al. 2000, Cobb 2002, Gilbert et al. 2005). Vertebrae, ribs, and teeth in the body are utilized because of their number, the rationale being that even if one is demolished, the collection of morphometric data will be unaffected. Additionally, teeth are thought to be extremely valuable as they are more robust than bones making them a good element for preserving DNA. However, as teeth are extremely valuable for stable isotope and morphometric analysis it is difficult to obtain permission to use them for DNA studies. The most commonly used bone for DNA extraction is the femur. Usually, a femoral head, a section of the mid-shaft, or a femoral fragment is removed, and sometimes exactly how or what part of the femur utilised is unspecified. As mentioned above, all of these methods are destructive to the data collection from the specimen. It is understandable that due to the destructive nature of the bone extraction method rendering specimens useless for further studies, there is reluctance on behalf of those responsible for skeletal collections to allow DNA studies involving the femur.

To ensure that the final DNA extraction does not have contaminants it is important to use decontamination standards from this level of the research. There are a number of criteria relating to the physical condition of bone that should be considered for the bone extraction including its destructive nature. Before conducting DNA analysis on skeletal material, it was necessary to devise a relatively non-destructive method of bone removal to carry out DNA research. The aims of this paper are; to describe the theory behind the bone extraction method, demonstrate its use in a pilot study, and finally to discuss this method in comparison to other published methods. Prior to describing the bone extraction method, an understanding of bone biology is necessary in order to understand the process of choosing the skeletal element and place of the bone extraction.

Bone Extraction Method

Bone Biology

The adult human skeleton is comprised of 206 individual bones, which itself constitute less than twenty percent of the weight of the entire body (White 2000). However, they are an essential mechanical component of the musculoskeletal system and also have important functions in protecting and supporting soft tissues. Furthermore, they are a living tissue that can repair and reshape themselves in response to external stresses. During life, bones produce blood cells as well as store fat and calcium. There are four classes of bones in the body: long bones (femur, humerus, tibia), short bones (metacarpals, metatarsals, phalanges), flat bones (scapulae, cranial bones, innominates), and irregular bones (vertebra, carpals, tarsals, cranial bones; Bass 1995). Long bones, like all bones in the body are described as a composite material formed of protein (collagen) and mineral (hydroxyapatite; White 2000).

Compact bone or cortical bone is the solid, dense layer that is found in the walls of boneshafts and on the external surfaces. In comparison cancellous or trabecular bone is spongy or porous, and has a honeycomb structure. It is found inside the compact layer particularly under protuberances, where tendons attach, in vertebral bodies, and at the ends of the long bones (Bass 1995, White 2000). As long bones are the main focus of this particular study, an understanding of their structural components is important.

Bone is a connective tissue composed of cells known as osteocytes and tis- sue basically has the same molecular and cellular composition in all mammals, which is relevant as the following statements apply to bone extraction methods from all mammals. The molecular and cellular composition of compact and cancellous bone are identical, differing only in porosity. In addition,bone is made up of both organic and inorganic material. The organic matrix gives bone its toughness and elasticity, while the inorganic material provides hardness and rigidity. The majority of the material in the bone matrix is inorganic and these are the minerals. This section of bone is made up of hydroxyapatite crystals (calcium phosphate), more specifically eighty five percent is calcium phosphate, ten percent calcium carbonate, the remainder is an admixture of calcium fluoride, magnesium phosphate, and sodium chloride (Bass 1995, White 2000). This mineral fills and mixes with the organic (collagen) matrix creating a weave of protein and minerals, which gives bone its structural properties. During life it is the organic matrix that allows nutrients and blood to flow through the bones. The organic matrix includes collagenous fibres, mucopolysaccharides (chondroitin sulphate and hyaluronic acid) and proteins (Coetzee 1987). Ninety percent of the organic matrix of bones is comprised of collagen (protein), which intertwine to form flexible and slightly elastic fibres in bone (White 2000). It is within the organic matrix of bone where cells and DNA are found. Post mortem the cells and DNA fuse to the inorganic structure of bone specifically the hydroxyapatite crystals.

Bone Extraction

Location on the Skeleton

Specific criteria emerged from the present research which determines the ideal place on the skeleton to retrieve a bone sample suitable for DNA analysis. Firstly, the bone extraction method should not interfere with any known morphometric sites used for individual reconstruction. The second criteria concerns the choice of bone. One with an ideal amount of the cancellous component should be utilized. Thirdly, the skeletal element must not be destroyed. Lastly, bone preservation should be considered as it is associated with the integrity of DNA (Haynes and Searle 2002). This is defined as the outward appearance of the bone based on softness, cracking and flaking. Damaged surfaces and lesions provide avenues for contamination from the environment into the bone. Therefore, it follows that no bone should be utilized with any damage or lesions on the external surface, or at the distal and proximal ends. Prior to bone extraction, the appropriate skeletal element must be chosen with the above considerations in mind.

Type of Bone

The first question is what kind of bone, compact or cancellous, should be utilized as this assists in reducing the options from the 206 bones available. Lee et al. (1991) state that cancellous bones can yield 10-to 20-fold more DNA than compact bone. The compact outer layer of bones protects DNA and therefore is usually less degraded after a certain period of time (Machugh et al. 2000, Wurmb-Scwark et al. 2003). Therefore, the chosen element must have a good yield of cancellous bone, as it is much easier to pulverize andis generally protected by robust compact bone. This rules out the ribs and smaller bones that do not contain enough cancellous bone, leaving the distal and proximal ends of the long bones as the final choice.

Bearing this in mind and in addition to the criteria listed above, it appears that the best area on the skeleton for the bone extraction is the distal end of the femur in the region of the intercondylar fossa. This region of the femur the space between the lateral and medial condyles has no known features of identification. In addition, there is a good store of cancellous bone in this area that is protected from environmental contamination by a hard layer of compact bone. The left side was chosen for all specimens,as the right side is more commonly used in metrical and morphological analysis.

How much is needed

In a study by Mays et al. (2002), it was suggested that 40-210 mg of bone powder is enough for DNA extraction. This was used as a guideline for the amount of bone necessary for successful DNA extraction in the present research.

Ideal method

Now that the best place on the skeleton for the bone extraction is known, along with how much bone is necessary for a successful DNA extraction, the bone extraction method needs to be considered.

Deciding on the methodological approach of the bone extraction was an experimental process which involved numerous procedures. Looking at the comprehensive literature written on the topic there were many considerations to explore. Contamination is an important issue prior to the extraction and during the bone extraction. Firstly, prior to the extraction the sample must be adequately prepared using strict decontamination measures to minimize the risk of a false positive result in the PCR (Yan and Watt 2005). Kemp and Smith (2005)

Suggest the use of bleach is the most cost-effective means for decontaminating the surface of bones. The surface should be washed with bleach and ethanol, then the first few layers physically removed, and if possible the material should be extracted from the internal surface (Kemp and Smith 2005). to be mechanically broken down to obtain a powder form that is easy to work with. The main concern when powdering the sample, is that extra manipulation increases the surface area for contaminant DNA molecules to bind (O'Rouke et al. 2000). The sample needs to be ground into a fine powder and this can be accomplished in many ways. The most commonly used method utilises liquid nitrogen with a pestle and mortar (Hagelberg et al. 1991, H¨oss & P¨a¨abo 1993, Cattaneo et al. 1995, Kolman and Tuross 2000). However, this method is not ideal not only did the bone disperse, but the final result was not a fine powder. In addition, the pestle and mortar was difficult to clean due to the fine grain of the composite material. Other researchers have used a commercially available bone mill or freezer mill, which also yields positive results (Hagelberg and& Clegg 1991, Meyer et al. 2000). However, this method was extremely expensive and not in the budget for the present research.

In this study, a simple, yet effective method of producing bone powder was accomplished using a sterile titanium bit attached to a power drill. After drilling, the bone powder was poured directly into a sterile container until further processing. This method not only decreases the amount of contamination but is also easily carried out and results in the formation of a fine powder, necessary for the current research.

This method of bone removal, for the purpose of DNA extraction from the distal end of the femur in the intercondylar fossa leaves only a small hole on the surface and does not interfere with known metrical or morphological points. While the use of this region ensures minimal destruction to the bone, it also provides enough bony tissue for the DNA analysis. Although this technique is marginally invasive it must be emphasized that it does not physically compromise the femur's use in future studies.

Pilot Study

Introduction

Once devised this optimised method was tested on 30 archaeological skeletons sourced from the Raymond Dart collection.

Materials and Methods

The sample was 30 archaeological skeletons sourced from the Raymond Dart Collection of human skeletons. This sample consisted of specimens that decomposed naturally that have no provenance associated with them, and so this sample was considered to be less valuable compared to material sourced from known provenance. Therefore, this was a good sample to test the method on and to demonstrate its use for more valuable material. Access to these skeletons was only granted after it had been shown that the method only left a small hole in the intercondylar fossa.

During every step of the preparation of the skeletal samples, similar precautions were taken that are usually followed for aDNA research (Hagelberg and Sykes 1989, Haas et al. 2000, Machugh et al. 2000, O'Rouke et al. 2000, Brown 2001, Mays et al. 2002). Protective clothing was worn, consisting of doctor's scrubs, latex gloves, hairnet, face mask and booties (Hagelberg et al. 1991, Kolman & Tuross 2000, Cobb 2002). In advance, drill bits and 50 ml tubes were autoclaved (Stericlav-28), a new bit and tube were used for each extraction. The tube was weighed before each extraction and the scale zeroed in order to give the accurate weight of the sample.

The extraction was performed under a sterilized flow form hood to protect the working surfaces. The extraction area was sterilized with 0.6% sodium hypochlorite followed by 70% ethanol, including all work surfaces and equipment (Hebsgaard et al. 2005, Kolman and Tuross 2000, Wurmb-Scwark et al. 2003, Wurmb-Schwark et al. 2004). Under the flow form hood paper towelling was placed over the cleaned working surfaces, the 50 ml tubes were labelled appropriatly and placed in a sterilised stand. Prior to the bone extraction, in order to remove any surface contamination the entire surface of the femur was wiped down with 0.6% sodium hypochlorite followed by 70% ethanol (Kemp and Smith 2005). Once dried, the surface in the intercondylar fossa was scraped with a scalpel to remove any trace of contaminant DNA, then subsequently wiped down with 70% ethanol (Hagelberg and Clegg 1991, Zoledziewska et al. 2002, Holland et al. 2003). The femur was held by the neck in the left hand, in a vertical position, while a power drill (Bosch PSB 650RE) with a sterile 4.5 mm titanium masonry drill bit was used to create a hole between the lateral condyle and medial condyle in the intercondylar fossa. Once through the cortical surface, a rotating action was used to break up some cancellous bone on the inner table to use for DNA extraction. The drill bit was withdrawn and the femur was inverted over the mouth of a 50 ml tube. The bone powder was gently tapped into the tube. This was done repeatadly until the weight of the bone powder was close to 1 g, 70 mg was needed for each DNA extraction. After the extraction, the extracted bone powder was stored for a short period of time at 4°C. In between each extraction gloves and surfaces were washed and wiped down with 0.6% sodium hypochlorite followed by 70% ethanol.

Conclusion

The result from the 30 samples was a fine to medium bone powder with an average yield of 0.85 g, which is more than adequate for DNA extraction. There was individual variation in the amount of the bone yielded, which could be attributed to the fact that bone mass decreases with the ageing process.

Discussion

Prior to conducting DNA analysis from skeletal material it is necessary to use a method of bone extraction that is minimally invasive to skeletal remains. Although this is a major concern for curators of skeletal collections, the bone extraction methods utilized in studies are not described in the literature. The majority just vaguely state that they have extracted bone powder. Therefore, it is important to have a published method that can be replicated in any laboratory.

In saying this there are other minimally invasive bone extraction methods available. Even some that have used drill like instruments, from dental drills to low speed electric drills (Greenwood et al. 1999, Faerman and Bar-Gal 1998, Adcock et al. 2001). The research papers that utilise drilling do so on different aspects of the skeleton, and on an inconsistent basis. There have also been methods that are minimally invasive using a dissolving method (Asher and Hofreiter 2006). However, the majority of these methods are not utilised on human remains, and as of yet there is no method that considers morphological and metrical areas on the skeleton.

Therefore, this method is ideal for analysis from human skeletal remains and it utilizes a novel area on the human skeleton in the intercondylar fossa of the left femur, which is not currently used for morphometric reconstruction. As a result the femur can still be utilised for future studies.

Future Applications

This method can be used to isolate any desired area on the genome to examine questions of demography and phylogeny. This bone extraction method has been utilised for molecular sex determination on the Raymond Dart collection of human skeletons.

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Mailing address: Victoria Gibbon
University of the Witwatersrand, 7 York Road
2193 Parktown, South Africa
gibbonv@gmail.com