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BONE CHIP, UV-C IRRADIATION AND LATEX GLOVES: How To Prepare A Bone Sample For Ancient DNA Analyses

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ABSTRACT

Within the last decade the young discipline of palaeogenetics has developed to a successful and expectant field of archaeobiological research. In the following article we give main advice on treatment and preparation of prehistoric bone samples when further analyses on molecular level are required. We demonstrate which precautions, working conditions, and monitoring protocols are necessary to avoid or at least minimize contamination with nucleic acids of any source. By following the catalog of proposed guidelines a major step towards the generation of authentic results (e.g. DNA sequence data) is done.

INTRODUCTION

The isolation and examination of the hereditary molecule deoxyribonucleic acid (DNA) from prehistoric and fossil bone samples has become one of the biggest challenges in anthropology within the past years. Traditionally, anthropologists use morphological features on human remains to directly assess information about the individuals' gender, age and the conditions under which they lived. Under good conditions of preservation of the bone morphology, statistical analyses of epigenetic variants (discreta) may allow anthropologists to make inferences about the genetic structure and thus, the kinship relations of prehistoric societies. However, it should be noted that a direct association between the genetic character of an individual and epigenetic variants has not yet been demonstrated (Scholz, 1996; Hauser and de Stefano, 1989). In addition, scientists out of different research fields have begun to analyze prehistoric bone material with alternative methods. The analysis of trace elements for example has been used to determine patterns in nutritional practices in prehistoric populations (Sealy *et. al.,* 1991). However, questions of kinship for example between individuals of a cemetery can be best addressed by the investigation of the fragmentary remains of residual DNA present in hard and soft tissues from archaeological sites (Hagelberg and Clegg, 1991; Scholz *et. al.,* 1997; Scholz *et. al.,* n.d.). Moreover, to determine the state of DNA survival within the hyaline collagen matrix of prehistoric bone, the investigation of the content of ancient amino acids and certain D/L values thereof is a necessary prerequisite (Poinar *et. al.,* 1996).

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Here we describe the handling of hard tissues (i.e. bone samples) derived from palaeontological specimens when further investigations on a molecular level are required. The guidelines described herein will help to meet a high quality standard to guarantee non-artefactual genetic analyses (e.g. DNA cloning, DNA amplification by the polymerase chain reaction (PCR)).

FORENSIC EXCAVATION

The condition of bone when found on an archaeological site depends not only on its biological properties, but also on its exposure before and during burial. The physical and chemical deterioration of bone is influenced directly by the conditions of soil, including acidity or alkalinity, degree of aeration, water movement and climatic changes. Bacterial action in well-aerated soils attacks the remaining tissues, mainly collagen and osteocalcin. Acidic soils attack the mineral salts and dissolution occurs at a rate dependant on the degree of acidity and water percolation (Chaplin, 1971; Cornwall, 1956). Since all actions mentioned also affect the molecular structure of nucleic acids in a more or less invasive manner, information available on burial conditions and the burial sites is important to the researcher, who may perform genetic experiments on a find. Thus, a detailed and accurate record must be kept of all site-specific data, the processes and materials applied to a specimen to help identify the correct answer to molecular genetic problems (Koob, 1984). However, very few people working on geological material actually document the environment as well as processes that a specimen can undergo, and most of the specimens have little if any associated documentation on past handlings, even if the excavation was in the last few years. Therefore, it is important that every process that a specimen undergoes is documented in painstaking detail; this includes casting and moulding, photography, gluing joints, washing, preparation, transportation and storage, besides the more obvious processes such as the dating of specimens or matrix, conservation treatment and repair of a find.

Exact documentation, however, does not preclude contamination occurring at the point of excavation and/or prior to the sample being passed for genetic analysis; it can only be relied upon to provide corroboration in conjunction with other methods.[2] For this reason “forensic excavation“ of finds has to become a more commonplace occurrence in future. To avoid possible contamination by the excavator it is necessary that latex gloves and face masks are worn during the excavation procedure. Unfortunately, this is rather the exception than the norm. For this reason several institutions and private excavation companies start employing specially instructed archaeobiologists; they introduce and survey all the necessary precautions to guarantee a “forensic recovery“ of prehistoric specimens.

At the best, material targeted for biomolecule (i.e. proteins, DNA) studies should be newly exposed bone without contamination of any kind. This includes common field materials (adhesives, consolidants, coatings, plaster and water) as well as contamination that can result from handling or from storage in archaeological environments. Even if it is not clear whether or not a specimen in the field will be studied in this way, field workers should remove an uncontaminated sample for future

[2] for more detailed information see paragraph ″authenticity″.

studies before proceeding with consolidation, adhesion or plastering. In addition, along with the find, a soil sample should be saved to facilitate reference experiments on the surrounding substrate. This may give informative insights on possible contamination with edaphone species, the mineral composition of the soil, and some important physico-chemical characteristics/features of the respective soil or sediment.

aDNA PRESERVATION

Encoded hereditary information specific to every individual (i.e. DNA polymorphisms and sequence variants) is stored in the mitochondria as well as the nucleus of every cell -and thus every sclerocyte- of the respective higher organism (Figure 1). After cell death, natural decomposition begins to degenerate the DNA macromolecule (i.e. polymer). The further decay of nucleic acids is dependant on weathering, soil chemistry and other biochemical reactions, such as hydrolyis or oxidation (Figure 2). Under certain and favourable conditions of preservation, various sized (low molecular weight) fragments of the original high molecular weight DNA may be preserved in bone. The presence of minute amounts of highly degraded nucleic acids requires the development of both sensitive and effective methods for the preparation of residual biomolecules out of prehistoric bone samples.

ASEPTIC CONDITIONS

In order to prevent possible contaminations when taking samples the following precautions are highly recommended: all working steps are carried out under sterile cauteles such as using a clean bench with laminar flow (Bachofer™), latex gloves, sterile working clothes, mouth masks and plexiglass face masks (Figure 3a/b). All appliances and containers used for working with or storing the bone material are cleaned from possible remaining bone powder residue in several steps (stone meal, acetone, propan-2-ol) before and after use. By irradiating the work area with UV-C light for approximately 2 hours it is free from DNA fragments of any kind. Decontamination of the containers and instruments used has to be carried out in two stages with substances capable of dissolving DNA and DNases (DNAaway, Molecular Bio-Products™, Inc., San Diego, CA).

SAMPLE PREPARATION

Certain areas of prehistoric skeletal remains are suitable for DNA extraction. Samples are mainly taken from the diaphysis region of the long extremity bones such as femur, tibia and humerus (Figure 4). Apart from the spongious substances unsuitable for use in the subsequent extractions these consist of solid bone substance, the Compacta. In it, because of its dense structure and under favourable preservation conditions, a sufficient amount of organic components (collagens) remain from which DNA may be isolated. On principle and on the condition that prehistoric DNA is preserved, every other skeletal bone sample (e.g. skull, clavicle) can also be used for the extraction of nucleic acids (Scholz and Pusch, in press).

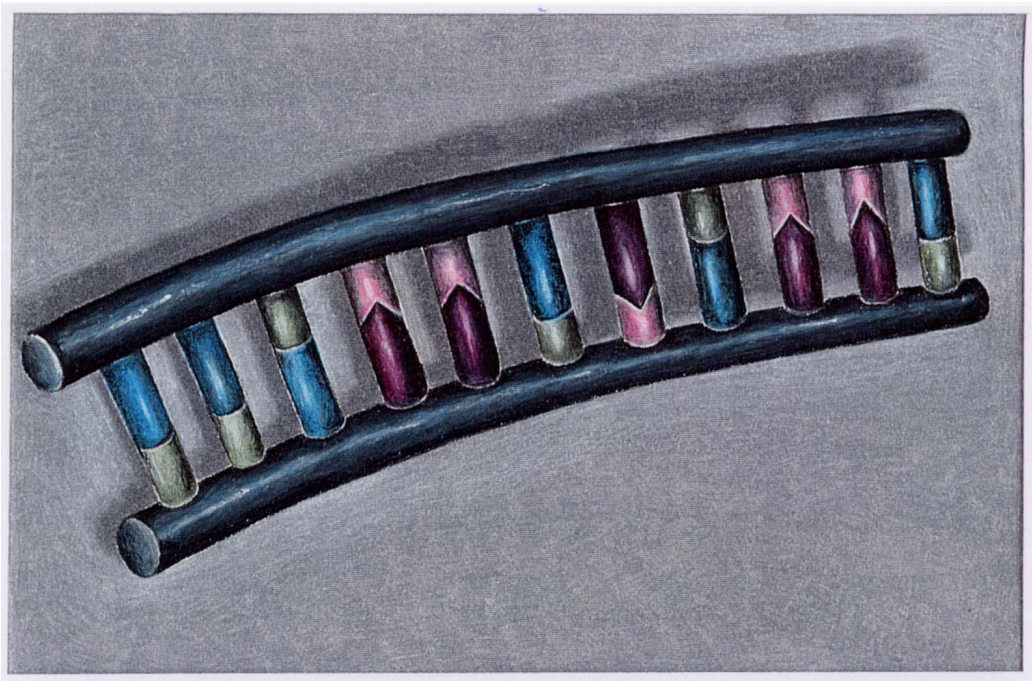

Figure 1. Schematic drawing showing the structure of an intact DNA molecule. Since DNA forms an inverted double strand (double helix), the strands are complementary to each other with respect to their base composition. The specific base pairing is between A:T and C:G (shown by different colours and morphology of the connection). (drawings: Claus P. Jakob, Nürnberg).

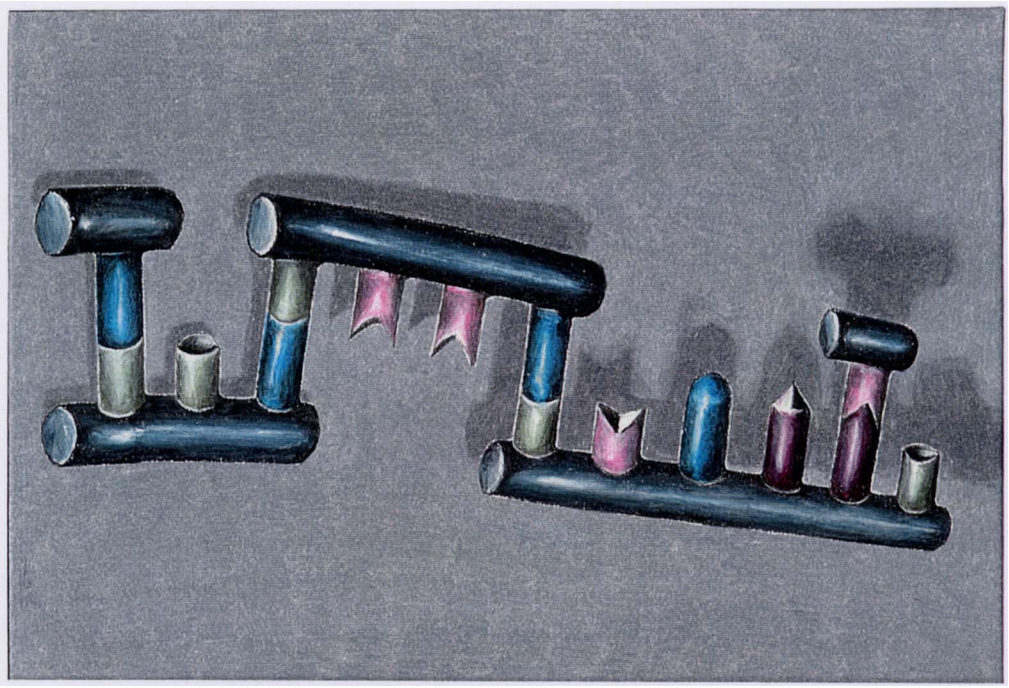

Figure 2. Different conditions of soil, weather and storage milieu may lead to greater or lesser amounts of degradation of the DNA over the course of time. This is indicated here by gaps within the molecule.

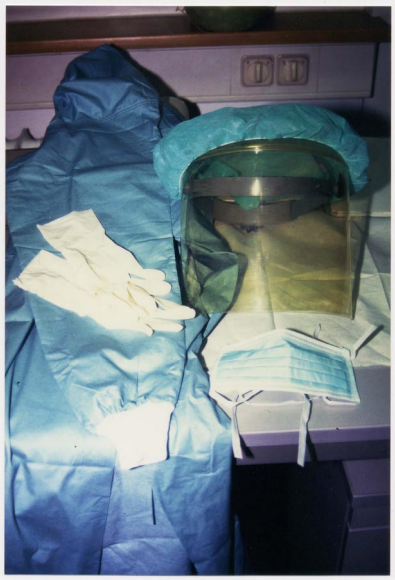

Figure 3a (above) and b (left). In order to guarantee further analyses under sterile conditions, special working areas (e.g. clean bench) and clothes (e.g. latex gloves and plexiglass face masks) have to be used in sample processing.

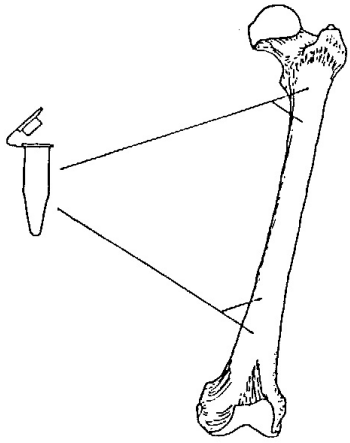

Figure 4 (right). Bone samples are mainly taken from upper and/or lower diaphysis regions of the long bones (e.g. tibia, humerus). Since the most prominent layers of the compact bone substance (Compacta) are located in these regions, they are specially applicable for attempting purification and subsequent DNA extraction.

At the best, the total sample consists of 2 to 3 bone fragments of about 1 to 2 cm^2 in order to make up for differences in usable DNA preservation because of the storage conditions. The bone chips are removed with the help of a hand mill (DBP Supra; Kaltenbach & Voigt/Biberach) which is normally used in dental clinics. In order to work as exactly as possible the mill has to be fitted with a diamond separation disc (Orthodontros™, Ultraflex 912-EF).

After the sample has been removed a slice of about 3 mm is milled off the sides of each sample in order to remove any contaminations and Spongiosa from the area of the Cavitas medullaris (Figure 5). For this work the mill has to be fitted with a new disc (Orthodontros™, milling head of plastic-diamond with 3-layer trianonlining, GD-No. 6830).

The bone samples are then mechanically ground into a fine powder with a vibration mill and sterile agate mortars (liquid nitrogen may be used as an alternative) in order to obtain the largest possible surface for subsequent lysis and extraction steps (Figure 6). After that, the bone powder is filled into a container with a screw-on top (Falcon BlueMax 2070) and gently mixed. From the sample size described about 6 to 8 g of bone powder can be obtained. A dry storage at room temperature is the most suitable condition for the bone powder, which is now readily available for further molecular genetic analysis (e.g. aDNA extraction, PCR, nucleic acids fragment cloning).

AUTHENTICITY

Paleogenetic investigation of ancient specimens is susceptible to falsification by the presence of contamination from more recent times. Contamination which can lead to amplification of non-authentic sequences is known to stem from several sources: (i) human biomoleculecules derived from the persons performing the genetic experiments, perhaps also from the archaeologists and other persons who have previously handled the specimens or (ii) edaphone DNA sequences derived primarily from bacterial or fungal growth upon the specimen. A third (and underestimated) source of contamination can arise from (iii) substances used for the restoration or conservation of specimens. Unfortunately it is still not standard practice to record the conservatory treatment of palaeontological specimens and the need for methods for the identification of contaminants arising from conservatory treatment has been recognized by several curators (Howie, 1984; Penning, 1976; Koob, 1984; Brothwell and Higgs, 1963; Carpenter, 1978; Fordyce, 1989).

Ad (i) and (ii): Characterization of the putative presence of any DNA (contemporary, vintage, ancient, sub-fossil or fossil nucleic acids) in (or attached to) a find is commonly tested by measuring the rate of amino acid racemization of aspartic acid (Asp), alanine (Ala) and leucine (Leu) in the sample (Poinar, 1996). When the D/L Asp values are smaller than 80/117 x 10^{-3} and are greater than those of D/L Ala and D/L Leu, no contamination of the sample with exogeneous DNA is likely, and the endogenous DNA might even be suitable for PCR amplification. Spectrophotometrical wavescanning analysis (240-500 nm) may further underline this conclusion (Scholz *et. al.,* 1998). Contamination of ancient DNA with vintage or

modern nucleic acids from the edaphon may also be excluded according to the procedure proposed by Pusch and Scholz (1997).

Ad (iii): Because of its cheapness and effectiveness, gelatine based glue[3,4] (also known by the German term "Knochenleim") has been commonly used for the preservation, repair and restoration of ancient or fossilized bone specimens from the early 19th century up to the present (Brommelle *et. al.,* 1984). This material however represents a rich source of non-authentic DNA. By means of southern hybridization experiments, we were able to demonstrate that bones treated with gelatinous glues contained DNA sequences typical for the particular species of animal from which the glue was prepared.[5] Even human DNA fragments were detected in several glues that were produced decades ago. Because of the similarity of glue used for conservation with degraded original collagen from the bone specimen, contamination is not readily recognized[6].

A sensitive method for the detection of contamination of ancient bone specimens with gelatine-based hardeners is highly recommended, and is based upon the elevated levels of D-serine and (to a lower degree) D-phenylalanine[7]. Generally, samples with D/L serine values outside the 2σ limits (95% confidence limits) can be considered as not belonging to the population of samples treated with glues. The lower 2σ limit determined from a number of treated samples (n = 14) was e.g. 91 x 10^{-3}. The D/L ratios for serine in non-treated specimens (n = 50), using subsurface sampling of the bone, scattered widely, with values of between 1.7 x 10^{-3} and 98 x 10^{-3} being found. In contrast, the maximum D/L serine value determined on surface samples of an untreated specimen was 32.6 x 10^{-3} (n = 14), which is well below the lower 95% confidence limit of 91 x 10^{-3} for treated sample populations. We therefore propose as criterion for probable contamination of bone samples with hardeners a D/L serine value higher than 91.9 x 10^{-3} measured on samples taken from the surface of the bone. Surface sampling has the further advantage of damaging less the ancient and usually valuable specimen; only approximately 1 mg of sample scraped from an inconspicuous area of the bone surface is required for the analysis.

[3] Shelton S.Y. and Johnson J.S. (1995) Conservation of sub-fossil bone. pp. 59-72; in: Collins C. (ed) The care and conservation of palaeontological material, Butterworth-Heinemann Ltd., Oxford.

[4] Lepper H.A. and Lewis G.E. (1941) Materials for preparation of vertebrate fossils: an analysis of their effectiveness. American Journal of Sciences 239, 17-24.

[5] unpublished data, Pusch and Scholz 1999.

[6] Glue-based hardeners are produced by the aqueous extraction and concomitant partial degradation of collagen from bones, sinews and hides. In a pre-treatment step prior to extraction of glue, the bones, sinews etc. are subjected to a prolonged treatment (up to 20 weeks) with an alkali (usually $Ca(OH)_2$) at ambient temperature. This treatment leads to increased racemization of the constituent amino acids, in particular those susceptible to racemisation under basic conditions, i.e. those with electron withdrawing groups neighbouring the centre of chirality, e.g. serine and aspartic acid.

[7] Approximately 1 mg of pulverized bone sample is hydrolyzed in 200 µl 6 N DCl in D_2O (24 h/110°C), esterified with 200 µl 1.5 N DCl in CH_3OD (15 min/110°C) and trifluoroacetylated (100 µl TFAA/10 min/110°C).The amino acid derivatives are dissolved in methylene chloride, separated by enantioselective gas chromatography on a Chirasil-Val capillary and detected by mass spectrometric selective ion monitoring. The D/L ratio of each amino acid is calculated directly from the respective peak areas. This general technique for racemization control has been described in more detail (Hodges and Smith,1994) and the analysis is commercially available (CAT, Gerhardt J., Heerstr. 2, 72074 Tübingen, Germany, Tel.: +49-7071-29-87618).

Figure 5 (left). Specific milling and separation tools used for the removal of contaminants possibly attached to the surface of a sample.

Figure 6 (below). Prehistoric bone powder stored in a sterile 1.5 ml Eppendorf reaction tube.

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