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Contamination controls when preparing archaeological remains for ancient DNA analysis

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Abstract

Contamination is of utmost concern when working with ancient DNA as it easily leads to false positive results. The best way to prevent or minimize contamination is to start precautionary measures as early as possible, ideally commencing with sample collection and preparation by field archaeologists. This paper discusses the nature of contamination in ancient DNA studies and offers some practical guidelines as to how archaeologists in the field can "clean-collect" samples for ancient DNA analysis. Methods for preparing contaminated samples from museum collections for ancient DNA analysis are also discussed.

Keywords: Ancient DNA; Archaeological remains; Contamination control; Sample preparation

1. Introduction

The analysis of ancient DNA provides archaeologists and anthropologists with innovative ways to study the past [14,15,22,28]. Since it was first applied to Egyptian mummified materials in 1985 [24], ancient DNA analysis of archaeological remains has generated many new insights into important archaeological and anthropological questions regarding human evolution and population affinities [7,17,23,31], kinship determination [12], diagnosis of human genetic and infectious diseases [19,32], domestication of animal and plant species [3,33] and subsistence practices of past human populations [20,27,36].

The applicability of ancient DNA to archaeology and anthropology, however, can be adversely affected by difficulties associated with the ancient DNA technique itself [11,16]. Besides DNA degradation in ancient remains that may leave no intact DNA molecules, contamination with modern DNA is of paramount concern as it can easily lead to false results. Contamination controls (i.e. strict precautions and measures) need to be exercised to prevent or minimize such contamination. Although there has been much discussion regarding contamination controls among ancient DNA researchers [4,16,26], specific information has not been made readily available [2] to field archaeologists and anthropologists who are often the first to collect and prepare human, animal or plant remains for ancient DNA analysis. Careful contamination controls during initial sample collection and preparation, however, can be extremely effective in minimizing the risk of false positive results.

This paper discusses the nature of contamination in ancient DNA studies, proposes a series of precautions for excavating and subsequent processing of ancient remains intended for ancient DNA analysis, and offers

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some suggestions for the proper preparation of previously excavated samples for DNA analysis.

2. Contamination in ancient DNA studies

Two major problems associated with the study of ancient DNA are the degradation of DNA over time and the contamination of ancient samples with modern DNA [11,15,22]. Physical and chemical degradation can destroy most of the DNA molecules contained in ancient remains. The minute amounts of preserved DNA are left in poor condition, damaged and broken into small pieces, causing difficulties in the analysis of ancient DNA molecules [18]. The invention of the polymerase chain reaction (PCR) technique in modern molecular biology [29] made it realistic to analyze ancient DNA [25]. The PCR technique is an extremely sensitive method that can detect minute amounts of specific DNA molecules, and amplify these molecules billions of times in a few hours [13]. PCR is an ideal tool for detecting ancient DNA molecules since theoretically a single molecule is enough to trigger PCR amplification. The hypersensitivity of the PCR technique, on the other hand, also allows for contaminant DNA to be amplified easily, generating false positive results if inadequate amounts of ancient DNA template are available. Although contamination concerns are associated with all PCR-based DNA analyses, including modern DNA work, due to the availability of adequate amounts of modern DNA molecules, the target DNA usually outnumbers the contaminant DNA. As a result, contamination may have insignificant or little impact on the analysis of modern DNA. However, due to the degradation of ancient DNA, the authentic template can be overwhelmed by contaminant DNA if effective contamination controls are not set in place [35]. As a matter of fact, contamination has been found to be responsible for the erroneously claimed positive PCR amplification of the alleged Dinosaur DNA and other million year-old DNA [1,10].

The high risk of contamination in ancient DNA studies can be better illustrated through the following example. While there are likely only a few thousand copies of mtDNA housed in ancient remains, one simple touch of the researchers' hand on ancient human remains can leave many shed skin cells, each of which can contain 1000 copies of mtDNA. In this case, contaminant human mtDNA easily outnumbers the authentic ancient DNA; PCR is more likely to amplify the contaminant DNA, resulting in false positive amplification.

Due to the increased risk of contamination in ancient DNA studies, the possibility of contamination must be thoroughly examined. The authenticity of the DNA must be carefully evaluated before ancient DNA results can be applied to answer archaeological and anthropological questions [16,26,36].

3. Sources of contamination

In a strict sense, the terms "contaminant DNA" and "contamination" in ancient DNA studies do not refer to the mixing of chemical impurities with ancient DNA samples, as they do in chemistry. Contaminant DNA refers to DNA that is identical or similar to the target ancient DNA which could be indiscriminately amplified by PCR. For example, bacterial and fungal DNA present in ancient human DNA samples (they make up the majority of total extracted DNA from remains [9]) should not be considered contaminant DNA if research targets ancient human DNA.

Sources of contamination vary considerably depending on the type of ancient remains and the types of research questions being posed:

1. In ancient human DNA studies, contaminant DNA can come from individuals who excavate, study and handle the remains as well as those who manufacture laboratory supplies such as chemical reagents and even test tubes [6,12,35].
2. For ancient faunal and floral DNA studies, contamination would most likely originate from modern reference specimens that are used for detailed one-to-one comparisons during morphological identifications of the remains [36]. Human DNA should not be considered a contamination source if distinctive PCR primers for ancient faunal and floral DNA studies are carefully chosen.
3. For ancient pathogenic DNA studies of bacterial species, contaminant DNA may also come from closely related species in soils and surrounding environments [8] (soil samples should therefore be collected in order to determine whether soils contain closely related species). PCR techniques should also be specifically designed to use those DNA markers that can distinguish target pathogenic species from possible contaminant species.

For ancient remains of certain antiquity (several thousands of years, for example), cross-sample contamination between ancient remains themselves may not be a severe problem since only a minute amount of DNA is preserved (at best) in the remains. Strict sample-to-sample controls, however, should still be in place as a precaution and also as a measure to stop surface contaminant DNA appearing on one sample from passing on to the next sample.

Obviously, the largest potential contamination source is the PCR products of previous PCR amplifications of the same or closely related species [11,12]. The

prevention of such contamination is wholly dependent upon the availability of a dedicated ancient DNA laboratory and the careful performance of experiments in an ancient DNA laboratory [26].

4. What ancient DNA researchers do for in-laboratory contamination controls

We use the term "in-laboratory contamination controls" to refer to the strict precautions and measures that are exercised in an ancient DNA laboratory. In spite of being seemingly directed towards ancient DNA researchers, we believe that the following discourse is useful to help our readers gain an understanding of the vigorousness of in-laboratory contamination control measures. We hope that this discussion will help field archaeologists appreciate the importance of pre-laboratory contamination controls in contributing to the overall success of ancient DNA studies.

For effective in-laboratory contamination controls, a dedicated ancient DNA laboratory is required: (1) to extract DNA from ancient samples (no modern DNA should be processed in this laboratory); (2) to prevent amplified PCR products from entering into the DNA extraction process and mixing with ancient DNA samples; (3) to prevent contamination from other samples that are being processed or those that have been processed previously in the laboratory; and (4) when working on ancient human DNA, to prevent ancient DNA laboratory technicians from shedding their own DNA into ancient DNA samples.

Several other precautionary measures should also be set in place to effectively implement contamination controls. In dedicated ancient DNA laboratories, ideally, equipment and rooms should be set up for the exclusive purposes of bone preparation, DNA extraction and PCR setup. PCR amplifications must be conducted in a physically separated PCR laboratory or area; this simple guideline has proven to be one of the most effective contamination controls [4,11]. In the ancient DNA laboratory, air should be UV-HEPA-filtered and positively pressurized, UV irradiation should be used to destroy possible DNA residues on bench and equipment surfaces after being wiped with bleach, and laboratory technicians should wear protective suits, gloves and masks to protect samples from being contaminated.

If all in-laboratory contamination controls are in place, the possibility of contamination during the ancient DNA lab process should be significantly reduced. Unfortunately, these controls cannot annul contamination that occurred prior to the ancient remains being sent to the ancient DNA laboratory. To a certain degree, most archaeological remains currently kept in museums and universities are contaminated [28]. When archaeologists excavated those remains 20 years ago, it is unlikely that they wore protective suites, gloves and masks, anticipating the advent of ancient DNA analysis in the future. Even if these remains were not contaminated during excavation, subsequent laboratory analyses of specimen-to-specimen comparison would likely have resulted in such contamination.

Such pre-laboratory contamination can be a serious problem for ancient DNA studies. As a "damage control", ancient DNA laboratories have had to use a variety of methods to remove contaminant DNA from ancient remains. These decontamination processes include physical removal of the contaminated surface of ancient materials or chemical destructions of contaminant DNA that may have penetrated into bone tissues [36]. Decontamination by nature is destructive and each method has different levels of effectiveness. Each specimen has a unique history of excavation and storage, and levels of contamination can vary considerably. Therefore, there is no means to guarantee complete removal of contaminant DNA through decontamination procedures. If decontamination is too destructive, it may also destroy too much of the authentic DNA that has fortunately survived to date. If decontamination is not adequate, contaminant DNA may not be thoroughly removed. Ancient DNA researchers must make balanced decisions concerning decontamination protocols and then implement complex research designs to identify contamination should it occur. The whole process is labor-intensive and time-consuming [16].

5. Pre-laboratory contamination controls by field archaeologists

5.1. Clean collection of samples during excavation

Effective pre-laboratory contamination controls by field archaeologists and subsequent lab workers can be most valuable in the success of overall contamination controls. Logically, contamination controls should be in place at the moment ancient remains are unearthed from the ground. Ideally, controls in the field should be as strict as the in-laboratory measures; however, outdoor excavation conditions may prevent such strict contamination controls. There are some general guidelines that should be followed during excavation and subsequent storage of samples that will minimize the chance of contamination.

1. Do not attempt to clean specimens designated for ancient DNA analysis, dirt on the specimens may serve as protection against contaminants entering into bone tissues, making in-laboratory decontamination easier.

2. Do not wash specimens as water may cause contaminant DNA to penetrate deeply into bone tissues and may also cause hydrolytic damage to ancient DNA.
3. If possible, avoid adding any preservatives to specimens as these chemicals may inhibit PCR amplifications and may cause potential contaminant DNA to adhere to the specimens [21].
4. Store specimens in cool, dry conditions to avoid further degradation of ancient DNA.
5. Store ancient specimens separately from modern reference specimens to prevent cross-sample contamination.
6. If possible, change gloves and clean or change tools from one specimen to another when handling. Specimens should be individually stored in plastic bags or tubes but only when they are completely dry. Otherwise, a paper bag should be used.

Field archaeologists and anthropologists should evaluate the possibility of collecting specimens for ancient DNA analysis in advance and prepare the required tools for clean collection. Unfortunately, it is unrealistic to expect that field archaeologists would be equipped with the same set of protective suits as ancient DNA technicians working in a dedicated laboratory environment. However, the use of common sense in clean collection should be applied. Generally, an ancient DNA sample collection "kit" should include disposable gloves, clean paper bags, aluminum foil, masks, hairnets, sealable plastic bags, bleach solution and clean excavation tools such as trowels and dental picks. For non-disposable tools, use a 10% commercial bleach solution for cleansing between samples (bleach has been found to be effective in "damaging" DNA [28]). (Unpublished data from our own lab and others indicated higher concentrations of bleach might be needed for more effective decontamination.) The clean collection tool-kit does not have to be sterile, but needs to be kept clean. Keep in mind that the same disposable tool should not be used for more than one sample or one skeleton.

An understanding of DNA preservation can also be useful when selecting ancient remains for DNA analysis [18]. Generally, ancient remains that date to within the last ten thousand years deserve serious consideration for ancient DNA analysis. However, in some cold regions, such as Arctic and Sub-arctic areas, materials for DNA analysis can be much older [30,34]. When selecting specimens, one should choose morphologically well-preserved specimens in the following order: teeth, cortical bone, and finally spongy bone. A small amount of bone (1–2 g) or one tooth is usually adequate for one ancient DNA extraction. For reproducibility tests, a second set of samples (bone or a tooth) should also be collected.

Special care should always be taken when collecting human samples for DNA analysis. If it is impossible for all personnel to take protective measures for the entire excavation, it should be reasonable for a couple of excavators to do so for the short periods of time required to collect the samples. Not all remains will undergo DNA analysis, therefore as a compromise, only a small number of samples need to be collected under strict contamination controls. In most cases, a few small bones (hand and foot bones, for example), fragmentary pieces of bone, or teeth should be sufficient for such analysis. However, due to the destructive nature of ancient DNA analysis, bones should not be selected if they also hold potential for morphological and pathological examinations [5]. If possible, multiple pieces of samples should be collected for reproducibility tests.

When the selected bones are collected, they should be wrapped in aluminum foil (not sealed) and put into a clean paper bag for natural drying. If wet, they should not be placed into a sealed plastic bag as moisture sealed in a bag will create ideal conditions for bacteria growth, which will cause further degradation to ancient materials and ancient DNA through endogenous (autolytic) and microbial nuclease activities. Ancient materials, however, can be sealed in a plastic bag such as Ziploc™ when materials are completely dry.

Burial environment can provide some useful clues about DNA preservation. If possible, one should collect other animal and plant species in addition to soil samples. This will allow for examination of soil chemistry which may shed some light on DNA degradation rates as well as the DNA preservation of other taxa. Although the main research focus may just be on ancient human DNA, DNA from other species collected at the site can be used later as secondary evidence for the authenticity of the human DNA [26]. If one succeeds in retrieving ancient human DNA but not animal DNA from the same site, the possibility of human DNA contamination should be examined with extra care.

When transporting the samples to the laboratory from the field, the designated specimens for DNA analysis should be stored in cool, dry conditions. For ancient human DNA analysis, researchers and previous field excavators should make their hair samples (with roots) or buccal swab samples available for DNA extraction and subsequent comparison with the studied human remains. These reference samples should also be sealed individually in separate clean test tubes.

When small bone elements or bone fragments are available, there is no need to cut them into smaller pieces if sample conservation is not a major concern. These bones can be simply shipped to an ancient DNA laboratory for sampling and the remaining portion can be returned. If a bone sample needs to be cut into smaller pieces, cortical bones should be chosen over

spongy bones; cortical bone is less porous, and therefore less vulnerable to contaminant DNA. The dense texture of cortical bones also provides a more protective milieu for DNA, making more ancient DNA molecules available for analysis.

5.2. Old materials from previous excavations

Previously excavated archaeological remains hold great potential for ancient DNA studies. Some of these samples may have been under intensive and extensive archaeological or anthropological studies prior to the decision to undertake ancient DNA analysis. The retrieval and analysis of ancient DNA from these remains may prove to be challenging due to the likelihood that most of these samples have been contaminated with modern DNA. For ancient human remains, it is difficult for one to trace the record of when they were handled and who studied the materials, making the collection of reference DNA samples from all previous researchers virtually impossible. This is an unfortunate reality, and it is essential to rely on ancient DNA lab technicians to conduct effective decontamination procedures inside the laboratory. A brief history of research conducted on the bone samples (such as when they were handled and how many people have handled them, and the accuracy of the records), if available, will be very helpful for ancient DNA technicians to decide which decontamination strategy should be used. Essentially, there is no need for field archaeologists or anthropologists to perform any decontamination measures since these practices, if not well controlled, may introduce new contaminant DNA into the samples.

The primary concern when selecting and preparing contaminated specimens for ancient DNA analysis is the avoidance of cross-sample contamination, specifically during the cutting of appropriate sample quantities. A small hacksaw should be used to cut long bones into small pieces of 1–2 cm. A new hacksaw blade should be used for each sample. Blades can be reused only after they are cleaned with detergent and wiped with a bleach solution. Dremel tools and drills can also be used and cleaned in similar ways, however low speeds should be used to prevent bone dust from spreading. The bench or table surface should be wiped with bleach solution and padded with new sheets of paper towel after every individual sample is processed. Each processed sample should then be individually stored in a sealable plastic bag, so long as it is dry.

6. Blind test

We advocate the implementation of blind tests in all ancient DNA studies (whenever possible) to aid in contamination detection [36,37]. Field archaeologists can help facilitate this objective. When preparing samples for ancient DNA analysis, all morphological and other identification information should be removed and the samples should be re-numbered; mock sample(s) (related species of animals and plants, or unrelated human skeletal remains) should also be incorporated into the sample set. The mock samples are those known samples (to archaeologists only) that are intentionally labeled as "legitimate" samples and mixed with study materials by archaeologists. Ancient DNA analysis should distinguish these mock samples from real study samples due to their anomalous DNA sequences. Success in doing so demonstrates the effectiveness of contamination controls and the reliability of ancient DNA analysis. The cross-examination resulting from the blind test is very informative for detecting contaminant DNA or for authenticating ancient DNA samples [36].

7. "Common sense works"

After consultation with field archaeologists, the authors have found that it is almost impossible to develop universal protocols that will suit all excavation situations and all types of ancient remains. As a result, this paper is not intended to provide highly specific and detailed procedures for contamination controls. Once the importance of contamination controls is realized and all possible contamination sources are identified, one can follow the "common sense" approach.

8. Other issues

1. This paper presents general guidelines for contamination controls for sample collection and preparation. However, field archaeologists are strongly encouraged to contact their potential ancient DNA collaborators for updated information and alternate measures for contamination controls.
2. The destructive nature of ancient DNA analysis may require ethical approval for DNA studies of human remains [15]. The collection of the comparative DNA samples also needs informed consents from crew members.
3. Ancient DNA preservation varies considerably from site to site, a pilot project of a few samples (3–5) should be conducted first to evaluate the state of preservation before a large number of samples are collected and prepared.

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